# The role of transcription factor dFoxO in regulating tracheal terminal cell plasticity of the fruit fly *Drosophila melanogaster*

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# Table of Contents

## Zusammenfassung

Die Umwelt, in der ein Organismus lebt, unterliegt raschen Veränderungen. Um mit diesen Veränderungen zurechtzukommen, müssen sich die Organismen an diese Veränderungen anpassen. Plastizität ist eine grundlegende Eigenschaft, die dafür sorgt, dass sich die Morphologie, das Verhalten und die Physiologie von Individuen an sich verändernde Umweltbedingungen anpassen. Das Verständnis derartiger plastischer Eigenschaften ist deswegen so wichtig, da eine Reihe von Erkrankungen des Menschen auf veränderte plastische Reaktionen zurückzuführen sind. Die Sauerstoffverfügbarkeit schwankt zusammen mit der Fülle der Nährstoffe während des Lebenszyklus eines Organismus erheblich. Ein verminderter $O_2$-Gehalt – bezeichnet als Hypoxie - kann zu irreversiblen Zellschäden führen, und daher haben Organismen adaptive Reaktionen entwickelt, um den $O_2$-Mangel auszugleichen. Die Fruchtfliege *Drosophila melanogaster*, ein weit verbreitetes genetisches Modell, ist sehr widerstandsfähig gegenüber Sauerstoffmangel, wobei das respiratorische System (Tracheen) der Insekten Gemeinsamkeiten mit dem Gefäßsystem von Wirbeltieren aufweist. Es ist bekannt, dass *Drosophila*-Larven bei Hypoxie eine Angiogenese-ähnliche Reaktion zeigen. Dabei wird die Expression von Zielgenen induziert, die zelluläre und physiologische Anpassungen an Hypoxie vermitteln. Die trachealen Terminalzellen (TTCs) sind von Natur aus plastisch und reagieren auf Hypoxie und unterschiedliche Ernährungsbedingungen, indem sie neue Verzweigungen aussprossen lassen, ein Prozess, der mit der Angiogenese verglichen werden kann. In der vorliegenden Arbeit steht die Rolle des Transkriptionsfaktors *Forkhead Box Protein O* (dFoxO) bei der Regulierung der Plastizität von Terminalzellen im Fokus. Die Lokalisierung von dFoxO in den terminalen Zellen unterscheidet sich von den anderen Zellen der Tracheen in ihrer Reaktion auf Hypoxie. Die Deregulierung der FoxO-Aktivität in den TTCs durch Mangel sowie durch Überexpression von FoxO führte zum Verlust der Plastizität der terminalen Zellen. Eine teilweise Verringerung der Expression von FoxO mittels RNAi führte jedoch zu einem Hyperbranching Phänotyp, was darauf hindeutet, dass FoxO auf einem physiologisch relevanten Niveau exprimiert werden muss, um die Plastizität der terminalen Zellen aufrechtzuerhalten. Die Blockierung der JNK-Aktivität in den terminalen Zellen hatte keinen Einfluss auf die Plastizität der terminalen Zellen, was darauf hindeutet, dass die FoxO-Regulierung in den terminalen Zellen unabhängig von dem JNK-Signalweg ist. Andererseits regulierte die induzierte Freisetzung von Insulin aus den insulinproduzierenden Zellen die terminale Verzweigung in positiver Weise, was darauf hindeutet, dass dFoxO über Insulin-Signale in den TTCs reguliert werden kann.

## Abstract

The environment in which an organism lives undergoes rapid changes, and to cope with these changes, organisms have to adapt. Plasticity is a fundamental biological process that ensures that individuals' morphology, behavior, and physiology adapt to match their environment. Understanding the mechanisms that regulate trait plasticity is important because knowledge of how phenotypic plasticity is regulated has significant consequences for the study of diseases that result from changes in plasticity. Oxygen availability, along with the abundance of nutrients fluctuates significantly during the life cycle of an organism. Reduced $O_2$ levels—or hypoxia, can lead to irreversible cellular damage in organisms, and therefore, organisms have evolved adaptive responses to compensate for the lack of $O_2$. The fruit fly Drosophila melanogaster, a widely applied genetic model, is highly resistant to oxygen deficiency and its respiratory (tracheal) system has features in common with the vertebrate vascular system. Drosophila larvae are known to display angiogenesis-like responses upon exposure to hypoxia. from where it induces the expression of target genes that mediate cellular and physiological adaptations to hypoxia. The tracheal terminal cells (TTCs) are plastic in nature, and they respond to hypoxia and different dietary conditions by sprouting out new branches, a process that can be compared to angiogenesis. Here we elucidate the role of the transcription factor, Forkhead box protein O(dFoxO) in regulating terminal cell plasticity. The localization of dFoxO in the terminal cells differed from the other cells of the trachea in response to hypoxia. Deregulation of FoxO activity in the TTCs achieved by deficiency and overexpression of FoxO led to the loss of plasticity of the terminal cells. However, partial reduction of expression of FoxO via RNAi led to hyperbranching phenotype, suggesting that FoxO need to be expressed at a physiologically relevant level to maintain plasticity of the terminal cells. Suppressing JNK activity in the terminal cells did not affect the plasticity of the terminal cells, suggesting that the FoxO regulation in the terminal cells is independent of JNK activity. On the other hand, the induced release of Insulin from the insulin producing cells positively regulated the terminal branching, implying dFoxO might be regulated via IIS signaling in the TTCs.

## List of abbreviations

| | |
|---|---|
| AEL | After Egg Laying |
| AGE-1 | AGEing alteration 1 |
| AKT | serine/threonine kinase 1 |
| AMPK | AMP-activated protein kinase |
| AMV | Avian Myeloblastosis Virus |
| ATP | Adenosine triphosphate |
| bHLH | basic helix–loop–helix |
| Bnl | branchless |
| Btl | breathless |
| cDNA | complementary DNA |
| ChR2 | channelrhodopsin-2 |
| CNS | Central Nervous System |
| Cora | Coracle |
| CRISPR/Cas9 | Clustered Regularly Interspaced Short Palindromic Repeats |
| CTCF | corrected total cell fluorescence |
| daf-16 | dauer defective-16 |
| DAPI | Diamidinophenylindole |
| DIC | Differential interference contrast |
| Dpp | Decapentaplegic |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxynucleotide Triphosphates |
| Dp | Drosophila phosphoinositide 3-kinase |
| DSRF | Drosophila serum response factor |
| EC | 5-ethynylcytosine |
| EGFP | Enhanced green fluorescent protein |
| ERK | Extracellular signal -regulated kinase |
| FACS | Fluorescence-activated cell sorting |
| Fga | Fatiga |
| FGF | Fibroblast growth factor |
| FGFR | Fibroblast growth factor recpetor |
| FOXO | Forkhead box, sub-group O |
| GFP | green fluorescent protein |
| GSK-3β | glycogen synthase kinase 3β |
| h | hour |
| hAEL | hours After Egg Laying |
| HIF | Hypoxia-Inducible Factor |
| HLH | Helix–loop–helix |
| HRP | horseradish peroxidase |
| HREs | hypoxia response elements |
| IGF | insulin/insulin-like growth factor |
| IGF1R | insulin/insulin-like growth factor 1 receptor |
| IIS | insulin–IGF-1 signaling |
| ILPs | insulin-like peptides |
| INTACT | isolation of nuclei tagged in a specific cell type |
| IPCs | insulin producing cells |
| IR | Insulin Receptor |
| IRS | Insulin receptor substrate |
| JAK-STAT | Janus kinase/ signal transducers and activators of transcription |
| JNK | c-Jun N-terminal kinase |
| MAPK | Mitogen-activated protein kinase |
| MARCM | Mosaic analysis with repressible cell marker |
| MEK | MAPK/ERK Kinase |

| | |
|---|---|
| MiMIC | Minos-mediated integration cassette |
| MMLV | Moloney Murine Leukemia Virus Reverse Transkriptase |
| mNSCs | median neurosecretory cells |
| mRNA | messenger RNA |
| MST1 | Macrophage Stimulating 1 |
| NESs | nuclear export signals |
| NM | Normal medium |
| ODDD | oxygen-dependent degradation domain |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline, Triton |
| PDK1 | phosphoinositide-dependent kinase |
| PFA | Paraformaldehyde |
| PHD | prolyl-hydroxylases |
| PI3K | Phosphatidylinositol-3-kinase |
| PIP2 | phosphatidylinositol (4,5)-bisphosphate |
| PKB | Protein kinase B |
| ppk | pickpocket |
| Pnt | Pointed |
| PolI | RNA polymerases I |
| PolIII | RNA polymerases III |
| PCR | Polymerase chain reaction |
| PtdIns3P/PIP3 | phosphatidylinositol (3,4,5)-trisphosphate |
| PTEN | Phosphatase and tensin homolog |
| PTMs | Post-translational modifications |
| RNA | ribonucleic acid |
| ROS | Reactive Oxygen Species |
| RTK | Receptor tyrosine kinase |
| sGCs | Soluble guanylyl cyclases |
| Shc | Src homology collagen |
| Sim | Single-minded |
| Sima | Similar |
| T1D | Type1 Diabetes |
| TBC1 | Tre-2, Bub2, and Cdc16. |
| TBC1D4 | TBC1 domain family, member 4 |
| TC | Terminal cells |
| TOR | Target of Rapamycin |
| TRAP | translating ribosome affinity purification |
| Trh | Trachealess |
| tRNA | transfer RNA |
| TrpA1 | Transient receptor potential cation channel, subfamily A, member 1 |
| TTCs | Tracheal Terminal Cells |
| TU | thiouracil |
| UAS | Upstream activating factor |
| Upd | Unpaired |
| UPRT | uracil phosphor ribosyl transferase |
| VEGF | vascular endothelial growth factor |
| VEGFR | vascular endothelial growth factor receptor |
| VHL | Von Hippel-Lindau |
| Wt | Wildtype |

**List of Figures**

## List of Tables

# 1 INTRODUCTION

## 1.1 Oxygen Homeostasis

Oxygen ($O_2$) is one of the essential elements required by living organisms to produce adequate amounts of energy necessary for metabolic activities to sustain life. Eukaryotes, including all multicellular organisms and some single-celled organisms, require a constant supply of oxygen for aerobic respiration to yield energy in the form of ATP [1]. Anaerobic organisms are exemptions and do not require molecular oxygen for growth. During evolution, various organisms have devised a wide array of different physiological and molecular adaptations for the transport and delivery of oxygen. In invertebrates, these possibilities range from pure diffusion to more efficient closed respiratory systems. Lower metazoans, like the flatworms and nematodes, can meet their aerobic oxygen demand solely by direct diffusion from the environment [2]. Insects have a more developed closed respiratory system with a mesh of fine tubular trachea that delivers oxygen to internal tissues and cells [3]. On the other hand, vertebrates have specialized organs for exchanging gases and cardiovascular transport systems to provide an increased diffusion interface. Vertebrates generally rely on respiratory pigments to transport oxygen to all body cells through the bloodstream [4]. In addition to transporting oxygen from the atmosphere to the cells, delivery systems require to adapt to oxygen availability to survive environmental variations.

Though oxygen is essential for aerobic life, the level of $O_2$, whether too low or too high, can be detrimental. In normal conditions, oxygen comprises 21% of the earth's atmosphere from sea level to the highest mountain top. Normoxia is a term used to describe the oxygen partial pressure ($pO_2$) that an organism usually lives in. Hypoxia is a condition when the $pO_2$ level falls below the normoxic $pO_2$, and there is no complete absence of oxygen. Hyperoxia is the opposite of hypoxia, a state where oxygen concentration exceeds normoxic levels and may lead to oxygen toxicity that causes extensive damage to tissues. The tolerance for fluctuating oxygen supply varies markedly among organisms, and the borders of hypoxia and hyperoxia are species or even population-specific [5]. In general, mammals are very intolerant to hypoxia, while fish, amphibians, and several invertebrates are more capable of tolerating hypoxia [6]. The air is considered oxygen-deficient or hypoxic for humans, below 19.5% oxygen, whereas *C. elegans* can maintain a near-normal metabolic activity at an environmental oxygen concentration of 2% [2]. Hypoxia can occur continuously or intermittently and be either acute

or chronic, depending on the duration [7]. The state of complete absence of oxygen is called anoxia. The condition of $pO_2$ of arterial blood being abnormally low is called Hypoxemia. Ischemia is a situation with blocked blood flow and subsequent restriction of oxygen supply to tissues.

In multicellular organisms, maintaining homeostasis requires the coordination of metabolic reactions and cellular processes with the constraints imposed by their environment. Most metazoan organisms need an uninterrupted and sufficient oxygen supply to meet their metabolic demand. Animals constantly monitor changes in the relative concentrations of both internal and environmental oxygen and carbon dioxide through chemoreceptors. In mammals, the carotid body is the main arterial chemoreceptor that senses reduced blood oxygenation. The carotid body controls ventilatory activity and induces robust arousal as a response [8]. In *C. elegans* and *D. melanogaster*, there is a specific subset of sensory neurons that expresses atypical soluble guanylyl cyclases (sGCs) that are capable of acting as oxygen sensors [9], [10]. They act as chemosensory centers to monitor oxygen gradient in the environment and respond to oxygen-regulated behavior [11]. Oxygen homeostasis is achieved by both rapid behavioral and long-term physiological responses, which involve changes in gene expression. In animal models, the most dramatic structural changes following hypoxic exposure are found in the vascular system, which undergoes remodeling to maintain a constant supply of oxygen to peripheral tissues.

## 1.2 Occurrence of Hypoxia in Animals

Oxygen supply can vary dramatically, with animals adapted to sea level, high-altitude, underground, and aquatic habitats. This variation poses a common physiological challenge to animals that obtain $O_2$ from the surrounding environment, from worms to mammals. Atmospheric hypoxia occurs naturally at high altitudes when the atmospheric pressure progressively decreases as the altitude increases. The lower air pressure makes it difficult for $O_2$ to diffuse into the vascular system resulting in $O_2$ deprivation, also known as hypobaric hypoxia [12]. Besides high altitude, many animals have to cope with temporary, permanent, or patchy hypoxic conditions during movement into a small den, cave environment, or confined space. In their natural soil habitat, *C. elegans* commonly encounter hypoxic conditions when the soil is flooded, blocking soil pore spaces or when $O_2$ levels would be low near areas of biological activity such as local concentrations of bacteria [13]. The larval and adult stages of

holometabolous insects experience very unique $O_2$ environments and face anoxic hazards during their life cycle due to their different habitats and locomotory capabilities [14]. Fishes inhabiting rivers and wetlands often come across hypoxia due to excess loads of organic material, nutrients, or rising temperatures resulting in low dissolved $O_2$ concentration [15].

Hypoxia in humans can result from several factors, including ambient hypoxia due to exposure to high altitude, physiological/transient hypoxia resulting from physical exercise, and pathological hypoxia due to disease conditions [16]. In situations where lowlanders remain at high altitudes for an extended period, it may eventually lead to mountain sickness [17], often associated with polycythemia, systemic and pulmonary hypertension, decreased exercise tolerance, decreased growth, and other hypoxia-associated symptoms. In the long term, ambient hypoxia can threaten humans' and animals' survival, development, and reproduction. In extreme cases, ambient hypoxia and mountain sickness lead to death [18]. Moreover, hypoxia is associated with many diseases where oxygen tensions are likely insufficient due to poor respiratory function, e.g., cystic fibrosis, chronic bronchitis, pulmonary hypertension, and Chronic obstructive pulmonary disease (COPD) [19]. Hypoxia also occurs in various tissues if there is elevated oxygen demand, e.g., in skeletal muscle during physical exercise or in case of tumor growth. Even under optimal conditions, oxygen availability can drop in the transition from rest to exercise, meaning that exercising skeletal muscle must function at a very low partial pressure of oxygen, resulting in hypoxia [20][21]. Tumors become hypoxic when there is an imbalance between the cellular $O_2$ consumption rate and the $O_2$ supply to the cells within the tumor [22]. Tumor hypoxia can be caused by inadequate blood flow in tissues due to abnormalities in the microvasculature, increased diffusion distances with tumor expansion, or tumor-associated anemic hypoxia resulting in reduced $O_2$ transport capacity of the blood. Tumors are well adapted to grow and expand in this persistently oxygen-depleted tumor microenvironment [23].

### 1.3 Rising to the challenge: Adaptation to Hypoxia

Animals exhibit diverse adaptations to survive reduced environmental oxygen. There are undeniably significant variations between different organisms and cells in their ability to survive in a hypoxic environment. Some of these strategies include the ability of organisms to slow down metabolism when exposed to hypoxia, shift to alternate pathways for energy production, enhance the $O_2$ carrying capacity, and increase the efficiency of gas exchange

under hypoxic stress [5]. Most of these processes occur during the early phase of hypoxia. They are activated through proteins that have already been synthesized. Still, these processes are also mediated by adaptive changes in the expression of genes encoding key factors of these responses [24].

There are high-altitude populations like the Andeans, the Himalayans, and the Ethiopians who live and work at heights of about 3,500–4,500m [25]. These populations have different phenotypes and strategies for coping with their inhospitable hypoxic environment [26], [27]. The Andean natives have 25-30% higher red cell mass, hemoglobin concentration, and hematocrit than the sea-level men [28]. By increasing the red cell mass, the hemoglobin concentration and $O_2$-carrying capacity of the blood is elevated. This polycythemia is one of the main adaptive mechanisms in men native to high elevations. It enables them to deliver oxygen throughout their bodies more effectively than people at sea level do. Tibetans compensate for low oxygen content by increasing their oxygen intake by taking more breaths per minute; that is, they have higher resting ventilation than people living at sea level [29], [30]. Tibetans also have lower lung nitric oxide levels than the Andean highlanders and lowlanders. Nitric oxide can act as a vasodilator and increase blood vessels' diameter, suggesting that Tibetans may offset low oxygen content in their blood with increased blood flow [31], [32].

Low $O_2$ availability is a physical stressor that leads to cell adaptations under physiological and pathological conditions. Hypoxia is dangerous for many animals because ATP levels fall because of decreased aerobic ATP production. When ATP levels fall, cellular homeostasis is quickly put at risk [33], and animals have developed different survival strategies to adapt to the situation. Animals typically respond to low oxygen tension shifting ATP production mechanisms from aerobic to anaerobic glycolysis [24]. To reduce energy consumption and maximize oxygen uptake, animals may try to slow down their metabolism and increase their respiratory volume. For example, in the turtle brain, energy-demanding processes are significantly suppressed to meet the energy supplied by anaerobic glycolysis, which includes decreases in excitatory neurotransmitter release, increased neural inhibition, channel arrest, and the suppression of action potentials [34]. To achieve increased respiratory volume in fishes, the gills undergo structural remodeling to maximize the oxygen uptake over the epithelial surface area of the gills [35]. During physical exercise in humans, hypoxia induces many responses in skeletal muscles due to rapid oxygen consumption. Chronic hypoxia reduces fiber area to improve oxygen diffusion into muscle cells and muscle mass to decrease oxygen

demand [36]. Acute hypoxia induced by exercise can lead to skeletal muscle vasodilation and hence maintain the delivery of $O_2$ as a compensatory response [37]. In *Drosophila*, low oxygen stimulates tracheal terminal branch formation. When the tracheal supply to a local region is eliminated, nearby terminal cells sprout branches that grow to supply the detracheated area. Oxygen-starved cells secrete a tracheogenic signal that can attract new terminal branches [38].

### 1.4 Molecular mechanisms underlying hypoxia response

Molecular oxygen was central to the evolution, being the core molecule to fuel energy production in the form of ATP through oxidative phosphorylation in aerobic organisms. Consequently, many organisms have become dependent on a constant supply of oxygen to function effectively during evolution. Organisms have developed molecular mechanisms to respond to hypoxia with the induction of genes encoding proteins that increase oxygen supply and modulate metabolic activity in oxygen-depleted tissue/organism [39]. The transcription factor, Hypoxia Inducing Factor (HIF), is the central regulator of oxygen detection and adaptation at the molecular level. HIF upholds oxygen homeostasis by regulating the expression of numerous genes and further initiates cellular responses to hypoxia by regulating glucose utilization and anaerobic respiration in hypoxic environments [1], [8]. One of the significant functions of HIF in mammals is to promote angiogenesis by regulating the vascular endothelial growth factor (VEGF) transcription. This central angiogenesis regulator leads endothelial cell migration toward a hypoxic area [40]. HIF also plays a crucial role in tumor masses surrounded by a hypoxic environment and enables tumor progression by inducing alternative metabolic pathways within cancer cells [41]. In addition, HIF has been shown to play a role in inflammation and hypoxia-induced apoptosis of various cell types [1].

HIFs are conserved throughout the metazoans and are present in genomes of organisms as primitive as nematodes, coral, and sea anemones to fish, amphibians, birds, and mammals. Three HIF-α subunits exist in mammals (humans and rodents): HIF-1α, HIF-2α, and HIF-3α, which heterodimerize with the ubiquitously expressed HIF-1β subunit, producing HIF-1, -2, and -3 [42], [43]. The lower Metazoans (corals, worms, and fruit flies as examples), which are thought to have evolved relatively early, have only one HIFα isoform most similar to human HIF1α. HIF is composed of two basic helix–loop–helix proteins (bHLH) — HIFα and HIFβ — of the PAS FAMILY (PER, AHR, ARNT, and SIM family) [44]. The β-subunit of HIF-1 is constitutively present, whereas the stability and transcriptional activity of the α-subunit is regulated by oxygen levels. HIF-1α is rapidly degraded in normoxic conditions [45], but low

oxygen tension stabilizes the HIF-1α protein. HIF1α undergoes proteolytic regulation dependent on the presence of an oxygen-dependent degradation (ODD) domain [46]. The alpha subunits of HIF are hydroxylated at conserved proline residues within the ODDD by HIF prolyl-hydroxylases (PHD), allowing their recognition and ubiquitination by the VHL(von Hippel-Lindau protein) E3 ubiquitin ligase, which labels them for rapid degradation by the proteasome [46]–[48]. Thus, under normoxic conditions, HIF is kept in a state of repression by the activity of the HIF hydroxylases. In hypoxic conditions, HIF prolyl-hydroxylase is inhibited since it requires molecular oxygen as a co-substrate [46]. Consequently, HIF-1α accumulates and migrates into the nucleus, forming a dimer with the HIF-1β component and thus becomes transcriptionally active. HIFα/β dimer binds to a specific DNA motif called the hypoxia response elements (HREs) [49], [50] and induces the expression of target genes that mediate cellular and physiological adaptations to hypoxia [44].

### 1.5 *Drosophila melanogaster* as a model system to study hypoxia response

*Drosophila melanogaster*, commonly known as the fruit fly or the vinegar fly, is a species of flies belonging to the family Drosophilidae with a phenomenal ability to colonize a wide array of habitats and environmental conditions [51]–[53]. *Drosophila* belongs to the group of holometabolous insects - that is, an insect with a larval and a pupal stage before the adult stage. The general body plan undergoes a dramatic reorganization during the process of metamorphosis during their life cycle. Their life cycle includes four stages: egg, larva, pupa, and adult. Larvae can move within the resource patch, while adults can fly between patches. Given that the life stages also included sessile status during egg and pupae, they exhibit adaptations against predation, parasitism, and environmental stressors, such as temperature extremes, ultraviolet light, and desiccation [54]. The entire life cycle is completed in about 12 days at 25°C; hence, many generations can be reared within a short period. In nature, fertilized females lay eggs into fermenting fruits or decaying organic material, and after complete maturation of the embryo, a first instar larva hatches from the egg. The hatched larva starts feeding and increases its weight by several folds while it molts twice to a second and a third larval instar. After 30 hours, the third instar larvae attain a critical weight and stop feeding. They move towards a relatively dry and clean area to become immobile and encapsulate in the pupal case, where they undergo a 4-day metamorphosis. While metamorphizing, the larval tissues are degraded to their basic components, although some larval organs are preserved. After the pupal stage, a fully formed adult or imago emerges from the pupal case (eclosion).

Adult male flies attain sexual maturity within hours of emerging, females don't have ripe eggs until two days after eclosion, and the cycle begins again.

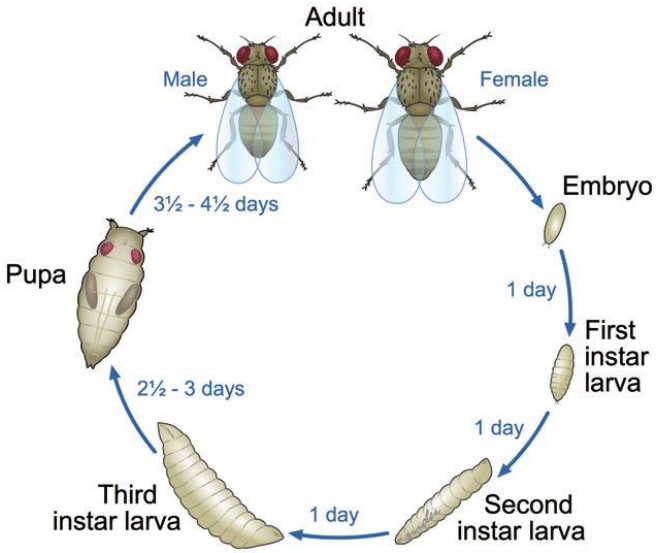



**Figure 1. The life cycle of the fruit fly *Drosophila melanogaster*.** The whole life cycle of the fruit fly *Drosophila* is relatively rapid and takes only approximately 10-12 days at 25°C. *Drosophila* development is divided into four stages: embryo, larva (first instar, second instar, and third instar), pupa, and adult.

Flies are well-developed genetic model organisms; they share numerous genes (~70%) and molecular signaling pathways with humans, making them apt to investigate many biological questions [55]. One of the most elegant examples of the methodological toolbox that allows to manipulate flies is the UAS/GAL4 system for targeted gene expression. The GAL4/UAS system allows for gene expression from any organism in a tissue and temporal-specific manner [56]. Mutations targeting most of the genes are available, and genes can be spatially and temporally overexpressed or silenced in almost any chosen pattern[57], [58]. Progress in *Drosophila* gene technology took a new leap, with the *Drosophila* genome fully sequenced [59]. The latest innovations like the MiMIC transposon system [60] and the CRISPR/Cas9 knockout/knockin and overexpression approaches [61] allow the inactivation, tagging, and overexpression of any gene in the genome within a short time.

The diverse habitats and locomotory capabilities of larval and adult holometabolous insects imply that they are exposed to varying oxygen environments and anoxic hazards during their life cycle [55]. *Drosophila* larvae primarily feed on decaying fruits and vegetables and the yeast that usually grows therein. In such a scenario, larvae compete with microorganisms for limited amounts of oxygen. Both adults and larvae are acutely tolerant to a low $O_2$ environment, withstanding hours of total $O_2$ deprivation (anoxia). For example, the brain of these animals

does not show any evidence of injury after a period of anoxia that can induce irreversible damage and death in rodents [62], [63]. Thus, *Drosophila*, being permanently exposed to low oxygen habitats, are well adapted with highly developed cellular machinery that responds to oxygen starvation [64].

### 1.6 Behavioral responses to hypoxia in *Drosophila* larvae

Previous studies on larval locomotion have shown that in the absence of food, larval movement is characterized by frequent stops and turns, which may be referred to as free wandering or exploratory behavior [65]. The behavioral response of freely moving larvae to hypoxia and hyperoxia can be studied by recording the number of stops and turns made by larvae crawling on agarose in a chamber containing different oxygen concentrations. When placed onto the plate in normoxia, larvae exhibit exploratory behaviors with a high rate (>20 min−1) of locomotory undulations. In approximately 5 min, larvae settle into feeding and begin interspersing feeding and locomotion. Under hypoxic conditions, larvae make a significantly reduced number of stops and turns, changing from exploratory to escape behavior. When exposed to anoxia, larvae cease feeding and crawl at a higher rate for the first 5 mins. For the next 5–20 min of anoxia, they show a higher rate of movement, but their locomotion rates decline with time. After 40 min of anoxia, larvae rarely move during any minute of observation [14].

### 1.7 The tracheal (respiratory) system of *Drosophila melanogaster*

The insect airway system provides one of the most effective gaseous exchange systems in the animal kingdom. Insects rely upon a branching network of air-filled tubes, the tracheal system, to deliver $O_2$ to the sites of respiration and carry $CO_2$ from the tissues to the atmosphere. The respiration system of the *Drosophila* larva is composed of two parallel dorsal trunks that span the dorsal side of the larva from the anterior to the posterior spiracles, enabling gas exchange with the external environment [66]. The spiracle function is regulated by the nervous system, which is sensitive to both $O_2$ levels in the tracheal network and $CO_2$ concentration in the hemolymph [67], [68]. The spiracles open through the cuticle and allow the entry of gases. The anterior spiracles remain closed until the third larval instar. This may be an adaption to the life conditions as the anterior half of the *Drosophila* larva is constantly immersed in the food medium. The airways of the fly have a simple architecture made of interconnected tubes

terminating in many fine blind ending structures (Fig.2). This highly branched tubular system is formed by an ectodermal monolayer epithelium around the central airspace. The diameter of the tubes decreases with proximity to the target cells and tissues so that the smallest tubules directly make contact with the tissues and cells and can be less than a micrometer in diameter [69]. The air-filled insect tracheal system facilitates a gas exchange route with a much higher diffusive capacity than water or blood, enabling flying insects to attain the highest mass-specific metabolic rates than any other animal [70]. The trachea is lined with a characteristic cuticle, the taeniolar forms minute spiral ridges. (Fig. 2). There are three levels of branching, classified based on different cellular mechanisms of tube formation. Primary branches are multicellular tubes that arise by cell migration and intercalation; secondary branches are unicellular tubes formed by individual tracheal cells; terminal branches are subcellular tubes formed within long cytoplasmic extensions [66]. Although the organization is less complex, it is a highly effective structure and shares numerous physiological similarities with the human lung [71].

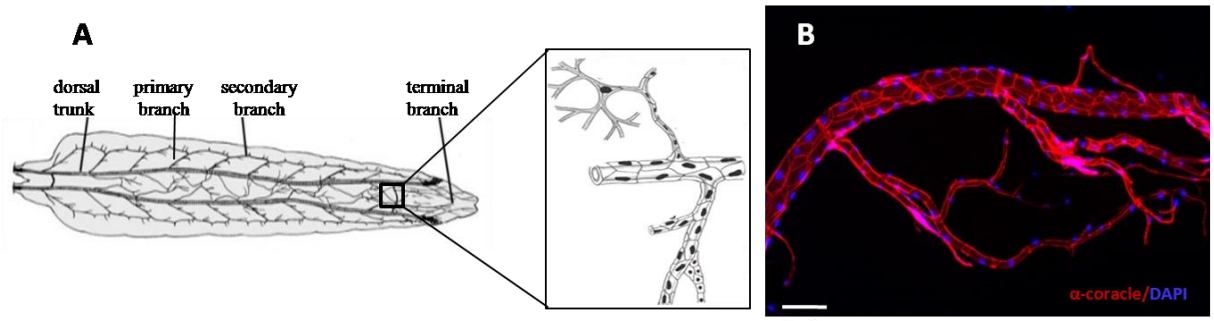



**Figure 2. The general architecture of the respiratory system of *Drosophila* larvae.** Structure of Larval trachea showing primary, secondary, and tertiary branches [66], [72]. B) Trachea of L3 larvae staining anti-coracle (red) antibody, which is specific for the membrane and with DAPI (blue) for the nucleus, scale 200 μm.

## 1.8 Mechanism of tracheal branching

The tracheal network in *Drosophila* begins as polarized epithelial cells that are formed during the cellularization of the blastoderm stage embryo. These epithelial cells form 10 sac-like invaginations, each consisting of approximately 80 epithelial cells on both sides of the embryo. Branches grow out of these invaginations. The entire branching process relies on cell migration, rearrangements, and cell shape changes in the complete absence of cell division through a highly orchestrated and reproducible manner. This structure generates the luminal cavity that

eventually expands and remodels during the branching process to form a complex network consisting of interconnected metameric units of different-sized tubes [73], [74].

Genetic screens of *Drosophila* larvae have revealed more than 50 genes involved in the formation of the tracheal system. Some of these genes are used during each generation of branch formation, whereas others are specifically involved in the early or late stages of the developing network. A gene expressed very early in development is *trachealess* (*trh*), a transcription factor, which appears in 10 patches bilaterally along the longitudinal axes of the larvae. This initiates the tissue locally to form slight invaginations (sacs) and defines cells as future components of the tracheal system[75], [76]. It also induces the expression of the gene, *breathless* in these sacs, which is the *Drosophila* ortholog of the mammalian fibroblast growth factor receptor (FGFR). The ligand for this receptor is Branchless (Bnl), the *Drosophila* ortholog of fibroblast growth factor (FGF) and is dynamically expressed in 6 patches of mesenchymal tissue around each tracheal sac. This diffusible factor induces bud formation and bud extension from the sacs resulting in 6 primary branches. Bnl initiates the cellular events that control and coordinate branch formation [77]. Breathless (Btl) expressed in developing tracheal cells can sense the source of Bnl. These tracheal cells adopt migratory properties and move towards the non-tracheal cells expressing a high concentration of Bnl/Fgf, which are in the vicinity while remaining attached to their tracheal neighbors [77], [78]. The process results in the formation of interconnected, bud-like extensions.

The high concentration of Bnl also induces the expression of *pointed* and *sprouty* at the tip of the branch. Pointed is a transcription factor that causes the tip of the branch to split into secondary branches, and Sprouty is an antagonist of Branchless that restricts branching to the tip by inhibiting branching further back along the extended branch. Secondary branches also express *pruned(blistered),* a transcription factor prerequisite for forming terminal branches. Blistered encodes the *Drosophila* serum response factor (DSRF), under the control of which the terminal cells differentiate and form tree-like structures of long, bifurcate, and hollow terminal extensions towards target tissues [79], [80]. An additional function of Bnl/Fgf signaling during the branching process is the activation of Notch signaling. An increased level of Fgf signaling in the tip cell of primary branches lead to the activation of the Notch (N) ligand Delta (Dl) [81]. Dl signals to the adjacent tracheal cells to inhibit them from also becoming fusion cells, thus limiting the number of fusion cells within each tracheal branch [82]. Both Bnl and Notch signaling cooperatively make cell fate choices during the branching progress and ensure that an accurate number of fusion and terminal cells are formed at branch tips [83].

Thus, *bnl* is turned on and off many times during tracheal branching. In summary, Bnl is used more than once during the branching process but at each level in a different context. At the level of primary branches, Bnl induces bud formation and extension; at the secondary level, high concentrations of Bnl promote the expression of genes involved in secondary branching; and at the level of terminal branches, Bnl promotes and directs the growth of oxygen-starved terminal branches.

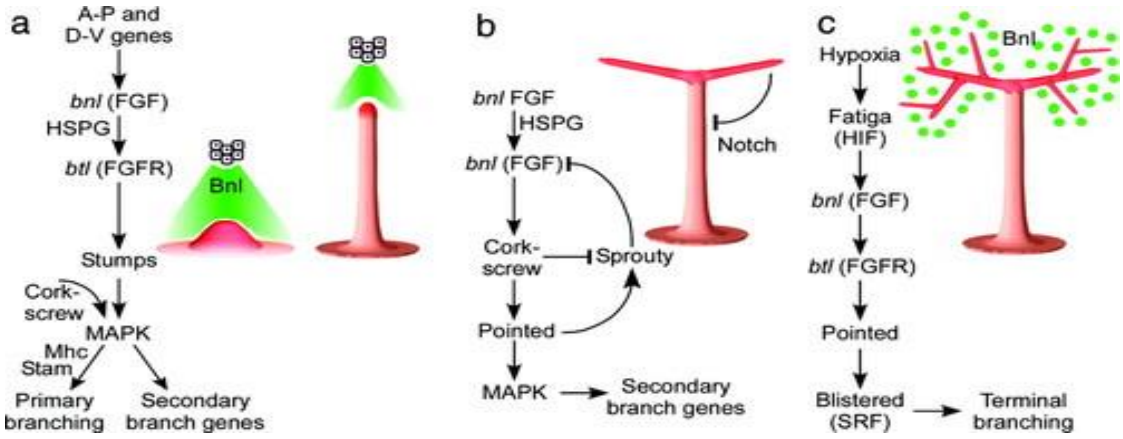



**Figure 3. Signaling pathways that regulate the branching of the *Drosophila* tracheal system.** a), The anterior–posterior and dorsal–ventral patterning genes induce *bnl* expression in mesenchymal cell clusters, which secrete and form a gradient of Bnl (green). Bnl induces *btl* expression (red) in the epithelial cells of the tracheal placodes that are closest to the Bnl source and acts as a chemoattractant. The migrating cells fuse into a primary branch. b) Bnl induces secondary branch tip-cell genes in epithelial cells sensing the highest Bnl signal, which then suppresses tip-cell gene expression in stalk cells via Notch signaling. Pointed is a pivotal tip-cell gene that upregulates MAPK signaling and Sprouty expression. Sprouty forms a negative-feedback loop by inhibiting Btl signaling. C) Hypoxia induces local expression of *bnl* via Fatiga, leading to Btl signaling via Pointed and Blistered and resulting in terminal branch sprouting [84].

## 1.9 HIF-dependentdent signaling in *Drosophila*

Fly gene homologs of mammalian *HIF-1α* are *trachealess (trh), single-minded(sim),* and *similar (sima).* As discussed earlier, Trachealess is a key regulator of tracheal development, whereas Single-minded is a protein responsible for controlling central nervous system midline cell specification [85]. Sima is most closely related to the human HIF-1α gene, demonstrating a conserved mode of regulation under hypoxia [86], [87]. An endogenous, oxygen-dependent HIF prolyl hydroxylase, highly homologous to the mammalian prolyl hydroxylase domains (PHDs), was discovered in *Drosophila* and was named *fatiga* [88], [89]. As in mammals, Sima has an ODD domain, which is the substrate of a *Drosophila* HIF prolyl hydroxylase and directs

oxygen-sensitive degradation[90]. The *Drosophila tango*(*tgo*) gene is reported to be highly related to the HIF-β in sequence, biochemical, and expression patterns [91]. Tango protein dimerizes with Sima through their HLH motifs and PAS domains to induce a transcriptional response to hypoxia [85], [92], [93]. Expression of Tango protein is ubiquitous in all tissues of the fruit fly throughout its development. Unless a subunit partner, such as Trh, Sim, or Sima is coexpressed in the same cell, Tango is primarily localized in the cytoplasm of all cells in the embryo. When expressed in the same cell, they translocate to the nucleus and initiate transcription of target genes(Fig. 2)[94].

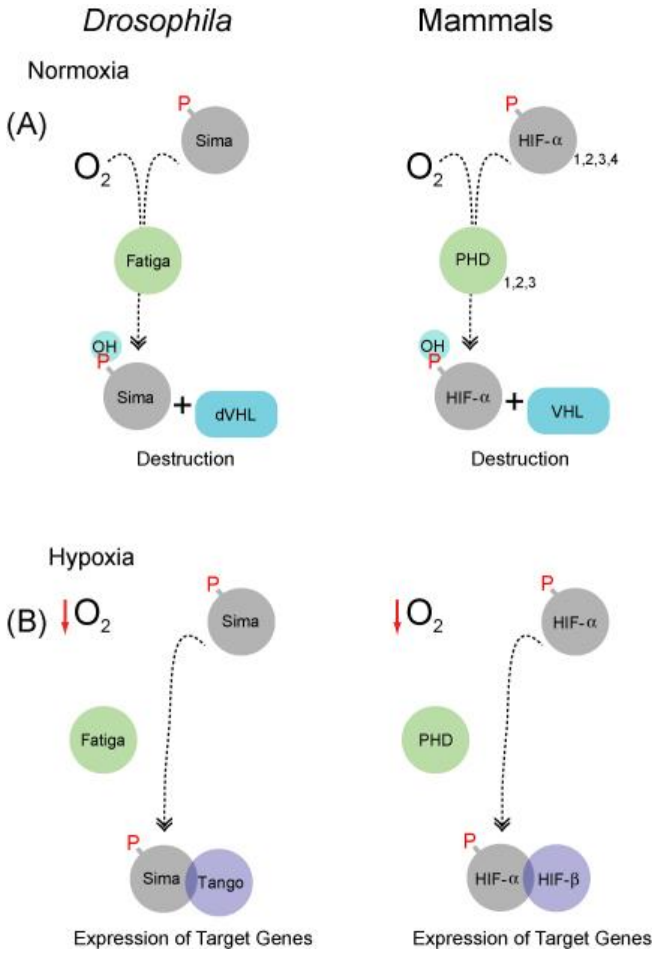



**Figure 4. Oxygen-dependent regulation of the stability of HIF-α proteins.** HIF-α in mammals or Sima in *Drosophila* are hydroxylated at specific prolyl residues in an oxygen-dependent manner by their respective specific prolyl hydroxylases PHDs in mammals and "Fatiga" in *Drosophila* (A) Hydroxylation of specific prolines of HIF-α proteins in normoxia enables interaction with the Von Hippel Lindau (VHL), which is the substrate recognition subunit of an E3 ubiquitin ligase enzyme. Interaction with VHL leads to HIF-α proteasomal degradation. (B) In hypoxia, VHL fails to interact with HIF-α due to the inhibited prolyl hydroxylation, resulting in stabilization of the protein, subsequent dimerization with the HIF-β subunit ("Tango" in *Drosophila*), and induction of HIF-dependent gene expression [95].

Sima shuttles continuously between the nucleus and cytoplasm. Nuclear import is facilitated by the nuclear localization signals (NLS) that map next to the Sima C terminus, and nuclear export is mediated via the two functional nuclear export signals (NESs) localized in the bHLH domain that is required for Sima steady-state subcellular localization and nuclear export upon reoxygenation [96]. Control of subcellular localization does not work as an all-or-none response but instead relies on oxygen levels in a dose-dependent manner and is regulated by developmental factors during embryogenesis. In flies, *sima* and its upstream antagonist *fga (fatiga)* play an essential role within tracheal terminal cells to regulate the process of extra branch sprouting induced by hypoxia. Ectopic activation of *sima* or deficiency of *fga* in the terminal cells can lead to excess branching, even in normoxia. These phenotypes have been attributed to the ability of *sima* to modulate the expression of the *breathless (btl)* gene, which in turn is activated by *branchless (bnl)* [88].

## 1.10 Tracheal remodeling and Plasticity of Tracheal terminal cells

In *Drosophila*, the adaptation to hypoxia involves mechanisms that increase oxygen delivery, such as the expansion of the spiracular openings, which can propel oxygen to the whole organism [158]. The expression of HIF1 increases the diameter of tracheal tubules and induces the expansion of cells that directly contact target tissues, the tracheoles [167]. Also, the *Drosophila* tracheal terminal cells exhibit plasticity and have the capacity to sprout out projections toward oxygen-starved areas in a process analogous to mammalian angiogenesis. The plasticity of terminal branching and the key role of oxygen in the process was first demonstrated in the large hemipteran insect *Rhodnius prolixus*. Blocking oxygen delivery to tissues led to a compensatory increase in terminal branching [97]. Oxygen-starved tissues in *Drosophila* produce a signal that controls tracheal branching. Rearing larvae under low oxygen causes increased sprouting of terminal branches throughout the animal, whereas high oxygen tension shows the opposite effect. Oxygen-starved tissues produce a local signal that induces tracheal sprouting and guides branches to the signaling source. Bnl was identified as a potent inducer of terminal branching that produces the local signal under low oxygen tension [98]. Taking into account the models for angiogenesis in mammals and the identification of Bnl as a potent inducer of terminal branching, the predicted model for *Drosophila* is that hypoxia is first sensed in target tissues, which would then turn on Sima-dependent induction of branchless, leading to tracheal extra-sprouting [92], [97], [99]. Contrary to the predicted model, tracheal cells showed expression of the hypoxia-responsive reporter with higher sensitivity than any

other tissue. More precisely, the tracheal terminal cells (TTCs) within the tracheal system are the most sensitive cells that respond to hypoxia [99].

GFP hypoxia-inducible transcriptional reporter. was first expressed in the trachea than in any other tissue, drawing an assumption that induction of tracheal specific genes is a reflection of a primary transcriptional response of the organism to hypoxia [3]. *Centanin et al*. determined that Btl is a key target of Sima in response to hypoxia. Forced over-expression of either Sima or Breathless in TTCs showed tracheal sprouting matching the phenotype observed in hypoxia. In contrast, suppression of the expression of Sima, specifically in TTCs, prevented tracheal sprouting. Thus, up-regulation of Sima in TTCs is necessary and sufficient for promoting tracheal sprouting in response to hypoxia [99]. Accordingly, TTCs autonomously respond to hypoxia by sending cytoplasmic projections to poorly oxygenated tissue. This autonomous response depends on the accumulation of Sima protein in the TTC, which in turn induces the expression of the FGFR, Btl in this cell. Up-regulation of the Btl receptor probably leads to increased sensitivity of TTCs to available levels of the FGF homolog Bnl from the neighboring tissue [99]. Nevertheless, Bnl is an important cue for terminal cell branching as its accumulation in the target tissues provides the necessary directionality to the outgrowth of tracheal branches [98].

Hypoxia was the only known environmental cue governing terminal cell sprouting until the recent past. Besides hypoxia, nutrition has been reported as an environmental factor that can induce TTC branching [100]. Linneweber *et al.* showed that high yeast concentration in the nutritional medium could induce TTC branching dorsal and specific branches targeting the gut. This effect relates to the sensitivity of TTCs to insulin-like peptides (ilp), secreted by insulin-producing neurons in the brain in response to nutrient availability [101]. Restricting the components of the insulin signaling pathway, insulin receptors, and phosphoinositide-3-kinase (PI3K) did not affect dorsal TCs but strongly reduced branches in gut-TCs. Larvae mutant for the InR ligands ilp2, 3, and, showed severely reduced branch numbers, while the TCs in most parts of the gut were unaffected. For those TCs that tracheate the hindgut, a neuronal population was identified that directly regulates TC branching in this region by secreting Ilp7. This suggested that dorsal TCs are more sensitive to the absence of the InR ligands than interference with their expression of InR. Yet, overexpressing InR in the trachea using a breathless driver increased dorsal TC branching by around 20% in another study [102].

## 1.11 Insulin/Insulin-like Signaling (IIS) Pathway

Insulin is the principal hormone controlling blood glucose and acts by stimulating glucose influx and metabolism in muscle and adipocytes and inhibiting gluconeogenesis by the liver. In addition, insulin/insulin-like signaling (IIS) modifies the expression or activity of various enzymes and transport systems in cells throughout the body. Besides the IIS pathway being critical for nutrient homeostasis, growth, and survival, efficient insulin signaling is also essential for healthy aging. Experiments with lower metazoans—*C. elegans* and *Drosophila* have shown that reduced insulin-like signaling extends life span. However, in higher animals—rodents and humans— reduced insulin signaling causes glucose intolerance and hyperinsulinemia that progresses to diabetes and reduces the longevity of the affected individuals [103].

In mammals, insulin is secreted by the β-cells in the Islets of Langerhans[104]. When the beta cells are appropriately stimulated, insulin is released from the cell and diffuses into islet capillary blood. The mammalian insulin-like signaling system includes three well-defined ligands—insulin, insulin-like growth factor 1 (IGF1), and insulin-like growth factor 2 (IGF2), that regulate the activity of the homologous insulin receptor (IR) and IGF1 receptor (IGF1R). By contrast, there are at least eight insulin-like peptides in *Drosophila* (DILP), produced mainly by a cluster of brain cells known as median neurosecretory cells (mNSCs), also known as insulin-producing cells (IPCs), where *dilp1*, *dilp2*, *dilp3*, and *dilp5* are expressed. These clusters are considered equivalent to the β-pancreatic islet cells that produce insulin in mammals [101]. The expression pattern of *dilp* genes is temporally controlled during development. For example, *dilp2* is expressed in all the stages, from embryo to adult, whereas expression of *dilp4* occurs during development before adulthood [105]. Animals with ablation of the IPCs displayed Type1 Diabetes (T1D) features and an increased level of circulating sugar compared to wild-type controls—the hallmark of diabetes. The increase in sugar levels that resulted from the ablation of IPCs was shown to be rescued by the expression of *dILP2* [106], [107].

The binding of insulin to the receptor initiates a cascade of phosphorylation events that activates enzymes that control many aspects of metabolism and growth. The IR belongs to the receptor tyrosine kinase (RTK) superfamily, which regulates a diverse array of cellular functions in multicellular organisms, including cell proliferation, survival, differentiation, migration, and metabolism [104], [108]. Upon ligand binding to the receptor, a complex

intracellular signaling network is activated through phosphorylation of the Insulin receptor substrate (IRS), which occurs in nearly all cells; still, other substrates exist in specific cells and tissues. There are different points of regulation or critical nodes, controlled both positively and negatively within the IIS pathway to ensure proper signal duration and intensity. The two canonical pathways of insulin signaling stemming from the insulin receptor-IRS node are the phosphatidylinositol 3-kinase (PI3K, a lipid kinase)/AKT (also known as PKB or protein kinase B) pathway [109] and the ERK (extracellular signal regulated kinase) pathway, also called the Raf/Ras/MEK/ MAPK (mitogen-activated protein kinase pathway) [110]. The PI3K pathway is accountable for most metabolic effects of insulin and is regulated solely through IRS, whereas the MAPK pathway is collectively regulated through both IRS and another substrate, Shc, and is involved in the regulation of gene expression and, in association with the PI3K pathway, in the control of cellular functions, including proliferation and differentiation [111]. In mammals, Akt phosphorylates many cellular proteins involved in various cellular events, including GSK-3β (glycogen synthase kinase 3β; the *Drosophila* orthologue is called shaggy), TBC1D4 (TBC1 domain family, member 4), the FoxO transcription factors and many more. Phosphorylation of FOXO by Akt leads to its retention in the cytoplasm, inhibiting its nuclear transcriptional activity [112].

Most of these interactions have also been well characterized in *Drosophila*. The IIS pathway of *Drosophila* consists of the insulin/IGF receptor (dInR), the insulin receptor substrate—CHICO, the *Drosophila* phosphoinositide 3-kinase Dp110/p60, and the PI3K target protein PKB. dInR conveys signals from *Drosophila* insulin-like peptides (DILPs) directly to PI3K or CHICO [113]. As one of the principal transcription factors in the insulin signaling pathway in *Drosophila*, dFoxO has a profound impact on animal metabolism. DILPs induce PI3K/Akt-dependent phosphorylation of dFoxO, facilitating its interaction with 14-3-3 protein, leading to nuclear exclusion and eventual ubiquitylation-dependent proteasomal degradation. Thus, Akt plays a key role in repressing dFoxO transcriptional activity in the flies [114].

## 1.12 FoxO Signaling

### 1.12.1 FoxO transcription factors

The forkhead box O (FoxO) family of transcription factors regulates diverse gene expression programs and affects many cellular processes, including cell cycle regulation, cell survival, and metabolism [115]. FoxO proteins are characterized by the presence of a conserved DNA-binding forkhead domain comprised of three α-helices and two long winged loops [116] and

mainly act as potent transcriptional activators by binding to the conserved consensus core binding site TTGTTTAC of the DNA [117][118]. The FOXO subfamily of Forkhead transcription factors is conserved from *hydra* to mammals. Bridge *et al*. first described the presence of a single FOXO gene in *Hydra*. In *Hydra*, it was shown that transient expression of FOXO-GFP protein induced an apoptosis rate of 20–60% in epithelial cells [119], [120]. In *C. elegans*, only the FOXO transcription factor is encoded by *daf-16*. Loss-of-function mutations in *daf-16* completely suppress the dauer-constitutive and longevity phenotypes associated with the reduced function of insulin-signaling components [121]. Based on knowledge about DAF signaling in *C. elegans*, forkhead transcription factors belonging to the FOXO subfamily have been identified as direct targets of insulin/IGF signaling in mammals. The mammalian homologs of DAF-16 are FoxO1, FoxO3, FoxO4, and FoxO6 [122], [123]. In *Drosophila*, the insulin-signaling pathway regulates the size of cells, organs, and the entire body in response to nutrient availability, by controlling both cell size and cell number and is mediated via dFoxO, the only *Drosophila* FoxO ortholog(dFoxO) [124]. FoxO proteins in *Drosophila, C. elegans,* and mice also govern neuronal morphogenesis and synapse plasticity, regulating downstream targets central to neuron development and physiology [125].

### 1.12.2 Regulation of FoxO Activity

FOXO transcription factors are regulated in response to external stimuli via post-translational modifications (PTMs) – phosphorylation, acetylation, mono- and poly-ubiquitination, methylation, and glycosylation. These PTMs modulate FoxO function in at least three ways: i) by altering FoxO subcellular localization (nucleus vs. cytoplasm); ii) by affecting FoxO protein stability; and iii) by modifying FoxO DNA-binding ability [126]. The external stimuli that regulate FoxOs include insulin, insulin-like growth factor (IGF), other growth factors, neurotrophins, nutrients, cytokines, and oxidative stress stimuli. Nucleocytoplasmic shuttling in response to external stimuli is the best-understood regulatory mechanism of FoxO function. Two evolutionarily conserved signaling pathways regulate FoxO activity by modulating the intracellular localization of FoxO proteins via PTMs. They may be loosely subdivided into two interconnected networks – growth factor signaling that promotes FoxO sequestration in the cytoplasm (negative regulation) and stress-activated pathways that generally facilitate nuclear translocation and transcriptional activation of FoxOs (positive regulation) [127].

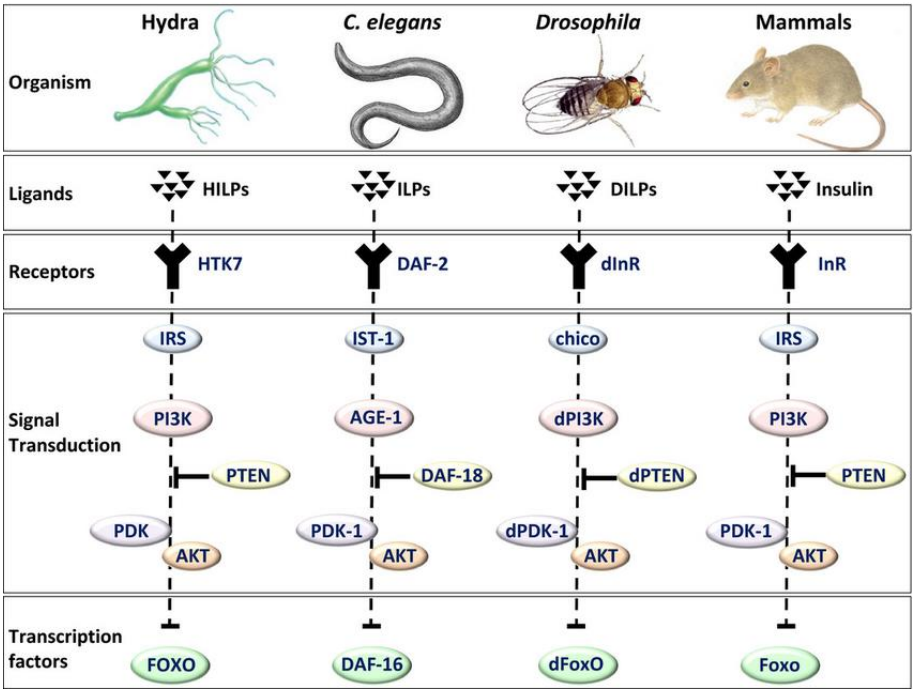



**Figure 5 . Insulin (INS) initiates the evolutionarily conserved IRS–PI3K–AKT signaling cascade to phosphorylate Forkhead transcription factor FoxO.** The corresponding orthologues of the significant components of the IIS pathway in *Hydra*, *C. elegans*, *D. melanogaster*, and mammals are illustrated. AGE-1, aging alteration 1; AKT, v-akt murine thymoma viral oncogene homolog 1; DAF-2, abnormal dauer formation-2; FoxO, forkhead family of transcription factor; INR, insulin receptor; IRS, substrate; IST-1, insulin receptor substrate (IRS)-like adaptor; PDK, phosphatidylinositide-dependent protein kinase 1; PTEN; phosphatase and tensin homolog.

From Hydra to mammals, growth factor-mediated regulation of FoxO transcription factors is mainly executed through PI3K (AGE-1 in *C. elegans*) and protein kinase B cassette. Insulin and growth factors act through PI3K to increase the levels of phosphatidylinositol (3,4,5)-trisphosphate (PtdIns3P/PIP3). These lipids act as second messengers to recruit and activate phosphoinositide-dependent kinase (PDK1) and PKB/AKT. PI3K-induced lipid formation is counteracted by the lipid phosphatase PTEN (phosphatase and tensin homolog). Active PKB translocates to the nucleus and phosphorylates FoxO at three conserved residues, resulting in increased binding of FoxO to the regulator 14-3-3 and cytoplasmic localization of both. On the other hand, FoxOs are activated in the presence of cellular stress through Jun N-terminal kinase (JNK) signaling and display increased transcriptional activity [128]. Following oxidative stress, when high levels of reactive oxygen species (ROS) are generated, FoxOs are activated through JNK-mediated phosphorylation and translocate from the cytoplasm into the nucleus [129]. JNK can antagonize insulin signaling at multiple levels by decreasing insulin receptor substrate (IRS) activity and inducing the release of FoxO from 14-3-3, thereby overcoming growth factor induced FoxO inhibition. Therefore, phosphorylation of FoxOs can both inhibit

(AKT/protein kinase B-mediated phosphorylation) and stimulate (c-Jun N-terminal kinase (JNK)-mediated phosphorylation) their transcriptional activity.

In *Drosophila*, the activation of dInR leads to the up regulation of a cascade of intracellular phosphorylation events, subsequently leading to the phosphorylation of the dFoxO protein. PI3K has a catalytic subunit, Dp110, and a regulatory subunit, Dp60, and functions to convert PIP2(phosphatidylinositol (4,5)-bisphosphate) to PIP3. The action of PI3K is antagonized by the activity of dPTEN, which reverses PIP3 back to PIP2. PIP3 acts as an intracellular second messenger that triggers a cascade of protein kinases, including dPDK1 and PKB/dAkt, leading to phosphorylation and nuclear exclusion of dFoxO. On the contrary, reduced insulin signaling through dInR or mutations in the substrate CHICO, or overexpression of the antagonist dPTEN, will lead to the shuttling of dFoxO from the cytoplasm to the nucleus, where it modulates the expression of genes that are involved in longevity and stress resistance. In flies, it has been shown that dFoxO extends lifespan when activated in the adult peripheral fat body. Remarkably, this limited activation of dFoxO reduces the expression of the insulin-like peptide dilp-2 synthesized in neurons and represses endogenous insulin-dependent signaling in pericerebral fat body, suggesting that insulin signaling operates in an autonomous and non-autonomous combine to control aging [113], [130].

Besides being phosphorylated by AKT and JNK, FoxOs can be post-translationally modified at various other residues. An attractive model of FoxO post-translational modifications has been proposed to serve as a combinatorial 'FoxO code', which can be recognized by binding partners to rapidly regulate gene expression, responding to various environmental stimuli [126]. For example, FoxO factors can be phosphorylated at diverse residues by several stress-responsive protein kinases, including AMPK, MST1, ERK, and p38 MAPK[131]. Similarly, several acetylated sites in FoxO may act as a 'molecular code' to recruit FoxO to different target promoters and/or to different protein complexes on promoters. For example, high levels of oxidative stress stimuli could trigger the recruitment of FoxO at promoters of target genes committed to apoptosis, whereas low levels of oxidative stress stimuli could lead to the recruitment of FoxO at promoters of DNA repair and ROS detoxification target genes [126].

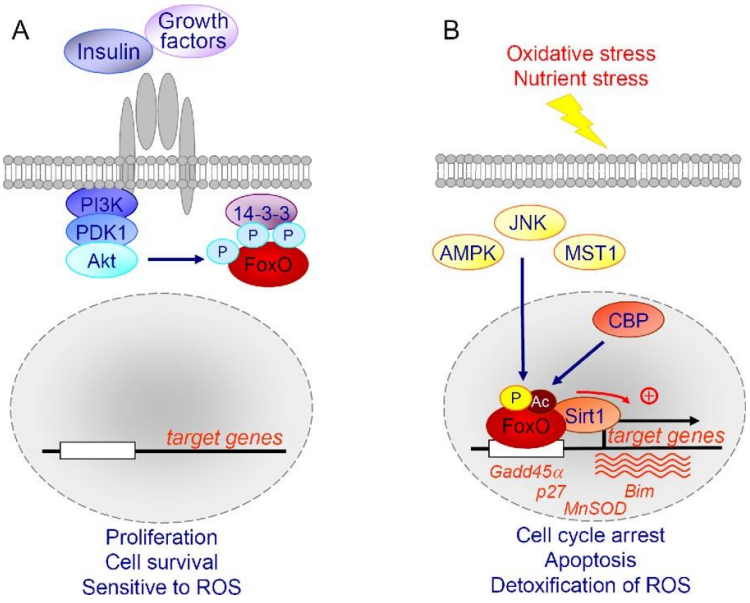



**Figure 6 . FoxO signaling.** Negative regulation of FoxO transcription factors by growth factors(A) and positive regulation of FoxO factors by oxidative and nutrient stress stimuli(B) [127].

### 1.12.3 FoxO as a regulator of plasticity at different levels of organization

Phenotypic plasticity, the ability for a single genotype to generate different phenotypes in response to environmental conditions, is biologically ubiquitous and leads to shifts in behavior and metabolism, as well as to changes in development, growth, and reproduction, which often enable organisms to improve the chances of survival and reproductive success. There are various studies where FoxO's role as a mediator of plasticity has been exemplified. *C. elegans* dauer diapause is a dramatic form of regulatory plasticity that demonstrates the links of the environment to life history traits. In *C. elegans*, DAF-16/FoxO plays a pivotal role in regulating vulval precursor cells' fate plasticity during dauer formation and for normal vulval patterning after passage through dauer, suggesting that DAF-16/FoxO links environment and life history with the plasticity of cell fate [132], [133]. Organisms cope with nutrition availability via developmental plasticity, reducing the size of some organs in response to the low nutrient variation while enabling others to develop nutritionally robustly. In a male fruit fly, the size of the genitals is resistant to dietary restriction. This is achieved by reducing the expression of FoxO in their genitalia. By lowering the production of FoxO, the genitalia can circumvent the hormonal signals that slow down the body's growth in response to low nutrition [134]. Recent studies have shown that FoxO proteins govern neuronal morphological plasticity by regulating downstream targets' central cytoskeletal dynamics and organization and the synaptic plasticity by regulating genes required at the synapse for neuron-specific processes, such as learning and memory [135]. Other examples include FoxOs in mammals, where these

proteins control skeletal muscle plasticity by modifying their structure in response to environmental change [136].

## 1.13 TU tagging

One of the essential hypoxic adaptations eukaryotic organisms make is the differential regulation of specific sets of transcripts. By varying the amounts of transcripts for various genes, cells can alter quantities of assorted proteins and, by doing so, change their physiology in ways that allow them to better cope with hypoxia. A deeper understanding of the changes in gene expression that occur during hypoxia will help identify therapeutic targets for diseases associated with hypoxia. Despite the availability of numerous molecular-genetic tools in flies to decipher gene function *in vivo,* it is still challenging to study gene expression in a tissue-specific manner or in a small population of cells [137]. Whole animal or even body-part-specific expression studies have significant limitations. For instance, in *Drosophila*, a significantly higher percentage of transcripts are identified when gene expression is examined in a tissue-specific manner compared to the entire organism [138]. This suggests that many rare or tissue-specific transcripts may go undetected when the whole organism or large body parts are used as starting material for these expression analyses. For instance, methods to isolate specifically the tracheal terminal cells are constrained by a limited cell population (only around 300 cells) and the fragility of these cells.

TU tagging entails cell type-specific expression of uracil phosphoribosyltransferase (UPRT) and administration of 4-thiouracil (TU), assuming that only cells expressing UPRT would incorporate 4-thiouracil into transcribing RNA. The TU tagging in *Drosophila,* is based on the transgenic expression of *Toxoplasma gondii* UPRT from a UAS promoter, which allows for tissue-specific incorporation of TU into newly synthesized mRNA when TU is fed to the adult flies or larvae [139]. After RNA isolation from the animals, only the mRNAs incorporating TU are coupled to biotin via the thiol-containing nucleotide and purified using streptavidin-coated beads [139]. TU-tagging has proven useful in several other systems[139]–[141]. However, given endogenous [142] and alternative [143] pathways for uracil incorporation, the labeling specificity in this method remains unclear. In addition, as demonstrated by Herzog *et al*. [144] and by Sharma *et al*. [145], labeling with TU-tagging of PolI and PolIII transcripts is ineffective, leaving tRNAs and ribosomal transcripts unlabeled.

## 1.14 Aim of the Study

Fluctuating environmental conditions are ubiquitous in natural systems, and biological organisms have evolved various strategies to cope with such fluctuations. Phenotypic plasticity refers to the capacity of the same organisms to exhibit different characteristics under varied environmental conditions, allowing organisms to express phenotypes better fitted to the environments encountered. This is often considered an adaptive strategy for living in varying environments. Here, we analyze a model of plastic adaptation in the tracheal system of *Drosophila melanogaster* larvae in response to varying environmental cues. The terminal cells of the tracheal epithelium in *Drosophila* are one of the few known cell types that undergo subcellular morphogenesis to achieve a stable, branched shape. Next to this cell-intrinsic branching mechanism, in this study, we examine the extrinsic regulation of terminal branching driven by the animal's environment. Hypoxia and nutrition availability are the two extrinsic environmental cues driving tracheal terminal cell plasticity in *Drosophila* larvae. Forkhead box O (FoxO) transcription factors are central regulators of cellular homeostasis and respond to a wide range of external stimuli, including growth factor signaling, oxidative stress, genotoxic stress, and nutrient deprivation. The primary focus of this study is to evaluate the role of FoxO in regulating the plasticity of the tracheal terminal cells in *Drosophila* larvae in response to oxygen and nutrition availability. Central to the FoxO regulation is the nuclear/cytoplasmic shuttling that determines FoxO activation. In this study, we aim to examine the effect of hypoxia on nuclear/cytoplasmic localization of FoxO in the trachea, focusing on the tracheal terminal cell. It is also the purpose of this study to examine if some of the hypoxia responsive phenotypes and metabolic consequences of hypoxia can be altered by regulating the expression of FoxO in the terminal cells. Various upstream regulatory inputs influence FoxO activity across multiple cell types to ensure appropriate downstream responses. Hence, the study also aims to analyze which major signaling pathways play a role in regulating FoxO in TTCs to regulate plasticity. Quantifying the differential expression of genes in tracheal terminal cell types is vital to understand a clear picture of molecular mechanisms by which cells sense and respond to hypoxia. Therefore, a further aim of this project is to analyze the use of a 4TU/UPRT-based biosynthetic labeling technique called the “TU-tagging” to isolate TTC-specific mRNA and prepare cell type-specific transcriptomes to gain insights into molecular mechanisms governing phenotypic plasticity.

# 2 MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Laboratory Devices

| | |
|---|---|
| Analytical balance (ABS) | Kern & Sohn GmbH (Balingen, Germany) |
| AxioImager | Zeiss (Oberkochen, Germany) |
| Balance (MXX-412) | Denver Instrument GmbH (New York, USA) |
| Bead ruptor 24 | Omni International (Kennesaw, USA) |
| Centrifuge (5415 D) | Eppendorf (Hamburg, Germany) |
| Centrifuge (5417 R) | Eppendorf (Hamburg, Germany) |
| Electrophoresis chambers | Biometra GmbH (Göttingen, Germany) |
| Geldocumentation (Transilluminator) | Heinrich Eimecke GmbH (Kiel, 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) |
| $O_2$ electrode (GOX 100) | Greisinger (Regenstauf, Germany) |
| pH 340/ION | WTW (Weilheim, 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) |

### 2.1.2 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) |
| Combitips (advanced, 10 ml) | Eppendorf (Hamburg, Germany) |
| Cover slips (24 x 24 mm) | Carl Roth (Karlsruhe, Germany) |
| Cover slips (24 x 50 mm) | Carl Roth (Karlsruhe, Germany) |
| *Drosophila* food vial (50 ml) | nerbe plus GmbH (Winsen, Germany) |
| *Drosophila* food vial (16 ml, 175 ml) | Greiner Bio-One (Kremsmünster, Austria) |
| Embryo collection container | Kisker Biotech GmbH (Steinfurt, Germany) |
| Falcon tubes (15 ml, 50 ml) | nerbe plus GmbH (Winsen, Germany) |
| Filtropour BT50 | Sarstedt (Nümbrecht, Germany) |
| Forceps | neoLab® (Heidelberg, Germany) |
| Gossamer, 150 µm | Eydam (Kiel, Germany) |
| PARAFILM® M | Sigma Aldrich (Munich, 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) |

### 2.1.3 General Chemicals

| | |
|---|---|
| Agarose | Biolab products (Bebensee, Germany) |
| Agarose (low-melt) | Biolab products (Bebensee, Germany) |

| | |
|---|---|
| Apple juice | Stute (Paderborn, Germany) |
| brewer`s yeast | Leiber (Bramsche, Germany) |
| Cornmeal | Mühle Schlingemann (Waltrop, Germany) |
| dNTPs | Promega (Madison, USA) |
| Dynabeads® MyOne™ Streptavidin C1 | ThermoFisher Scientific |
| EZ-Link™ HPDP-Biotin | ThermoFisher Scientific |
| Molasses | Biohof Heidelicht (Gerdau, Germany) |
| Methyl 4-Hydroxybenzoate reagentplus® (nipagin) | Sigma Aldrich (Munich, Germany) |
| Normal goat serum | Sigma-Aldrich (Munich, Germany) |
| PFA | Polysciences Inc. (Warrington, USA) |
| Sugar beet syrup | Kanne Brottrunk (Selm-Bork, Germany) |
| Xylene cyanol FF | AppliChem (Darmstadt, Germany) |
| Yeast extract | BD (Franklin Lakes, USA) |
| 4-Thiouracil | ThermoFisher Scientific |

### 2.1.4 *Drosophila* fly lines

**Table 1. Fly lines and their genotypes used in this study**

| Name | Genotype | Vendor |
|---|---|---|
| w1118 | w[1118] | BDSC: 5905 |
| DSRF-GFP-Gal4 | DSRF-Gal4;UAS-GFP | (Gervais and Casanova, 2011) |
| DSRF-Gal4 | DSRF-Gal4; | (Gervais and Casanova, 2011) |
| ppk4-Gal4 | yw67c23;pkk4-gal4;+/+ | Generated from Christina Wagner |
| btl-Gal4 | w-; btl-GAL4, UAS-GFP/Sp; TM2/TM6b | Leptin Group, Heidelberg |
| UAS-GFP | y1 w*; wgSp-1/CyO, P{Wee-P.ph0}BaccWee-P20; P{20XUAS-6XGFP}attP2 | BDSC: 52262 |
| UAS-FoxO in II | y[1]w[*];P{w[+mC]=UASfoxo.P}2 | BDSC: 9575 |
| pUAS-FoxO-GFP | w[1118]; P{UAS-foxo-GFP} | BestGene (Diss. C.Wagner) |
| UAS-ChR2-XXL | y[1] w[1118]; PBac{UASChR2.XXL}VK00018 | BDSC:58374 (Dawydow et al, 2014) |

| | | |
|---|---|---|
| UAS-UPRT3 | w[*]; P{w[+mC]=UAS-UPRT.M}3.1 | BDSC: 27602 |
| UAS TRPA1 | w[*]; P{y[+t7.7] w[+mC]=UAS-TrpA1(B).K}attP16 | BDSC: 26263 |
| UAS-basket$^{DN}$ | w[*]; P{w[+mC]=UAS-bsk.K53R}20.1a | BDSC 9311 |
| FoxO-RNAi | y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF02734}attP2 | BDSC: 27656 |
| thor-LacZ | y[1] w[*]; P{w[+mC]=lacW}Thor[k13517] | BDSC:9558 |

2.1.5 Antibodies and dilutions.

**Table 2. Antibodies, dyes, and their corresponding dilutions**

| | Dilution | Vendor |
|---|---|---|
| **Primary antibody** | | |
| α-Coracle, mouse (C615.16) | 1:500 | DSHB (Iowa City, USA) |
| **Secondary antibody** | | |
| α-mouse-AlexaFluor 555, goat | 1:500 | Cell Signaling Technology (Danvers, USA) |
| **Fluorescent dyes** | | |
| Roti®-Mount | | Carl Roth (Karlsruhe, Germany) |
| FluorCare DAPI (4,6-diamidino-2-phenylindole) | | Carl Roth (Karlsruhe, Germany) |

2.1.6 Oligonucleotides

**Table 3. Primer sequences used for PCR**

| Primer | Oligonucleotide Sequence |
|---|---|
| OdT-T7I | GAGAGAGGATCCAAGTACTTATACGACTCACTATAGGGAD AT(25)V |
| Rpl32_For | CCGCTTCAAGGGACAGTATC |
| Rpl32_Rev | GACAATCTCCTTGCGCTTCT |
| DSRF_For | TACACGACCTTCTCCAAGCG |
| DSRF_Rev | GTTGAGGCAGGTCTGGATGA |
| DH44_For | TGGAACACGGAACTCACAGG |
| DH44_Rev | GATTCAGTTCGACCTGGCGG |
| OA2_For | GGCAACGAGTAACGGTTTGG |
| OA2_Rev | TCATGGTAATGGTCACGGGC |
| OAMB_For | CTGCCGTGAGAACGACGAG |
| OAMB_Rev | GCGCAATATGAGCTGGGACT |

### 2.1.7 Enzymes and Kits

**Table 4. List of Enzymes and kits used**

| | |
|---|---|
| Pwo DNA Polymerase | Roche (Basel, Switzerland) |
| SuperScript™III Reverse Transkriptase | Invitrogen, Darmstadt, Germany |
| Taq DNA Polymerase F100 L | Thermo Scientific (Waltham, USA) |
| GeneRuler 1 kb DNA Ladder | Thermo Scientific (Waltham, USA) |

### 2.1.8 Solutions and Buffers

Phosphate buffered saline (PBS):

| | | |
|---|---|---|
| 136 | mM | NaCl |
| 2.7 | mM | KCl |
| 1.5 | mM | $KH_2PO_4$ |
| 7 | mM | $Na_2HPO_4$ |

Ad with $H_2O$, adjust to pH 7.3 with HCl, autoclave

Hemolymph-like solution (HL3):

| | | |
|---|---|---|
| 70 | mM | NaCl |
| 5 | mM | KCl |
| 1.5 | mM | $CaCl_2$ x 2H2O |
| 20 | mM | $MgCl_2$ x $6H_2O$ |
| 10 | mM | $NaHCO_3$ |
| 5 | mM | trehalose |
| 115 | mM | saccharose |
| 5 | mM | HEPES |

Ad with H2O, adjust to pH 7.1 with NaOH, filter sterilize, storage at 4 °C

Tris-borate-EDTA (TBE) buffer:

| | | |
|---|---|---|
| 89 | mM | Tris-HCl |
| 89 | mM | Boric acid |
| 2 | mM | EDTA |

Ad with $H_2O$, adjust to pH 8.0 with HCl, autoclave

Tris-EDTA (TE) buffer:

| | | |
|---|---|---|
| 89 | mM | Tris-HCl |
| 2 | mM | EDTA |

Ad with $H_2O$, adjust to pH 8.0 with HCl, autoclave

DNA gel loading dye (10x):

| | | |
|---|---|---|
| 500 | µl | glycerol |
| 500 | µl | $H_2O$ |
| 500 | µl | 1 % (w/v) bromphenol blue |
| 500 | µl | 1 % (w/v) xylene cyanol FF |

Fixation solution:

4% (w/v) paraformaldehyde (PFA)

ad with PBS, heat to 50-60 °C until complete dissolution, storage at -20 °C

Washing buffer (PBST):

0.1 % (w/v) triton X-100

Blocking buffer:

10 % (v/v) normal goat serum ad with PBST

10 mM sodium phosphate buffer

| | | |
|---|---|---|
| 10 | mM | $Na_2HPO4$ |

Solution for fixation

0.75% Glutaraldehyde

Staining solution

| | | |
|---|---|---|
| 150 | mM | NaCl |
| 1 | mM | $MgCl_2$ |
| 3.1 | mM | $K_2[Fe(CN)_6]$ |
| 3.1 | mM | $K_3[Fe(CN)_6]$ |

| | | |
|---|---|---|
| 0.3% | mM | Triton X-100 |

In 10 mM sodium phosphate buffer

10 % x-GAL stock solution solve

89 mg in 890 µl DMSO (stored at -20° C)

X-GAL staining

25 µl x-GAL in 1 ml staining solution

### 2.1.9 Standard fly food

Normal medium (NM), 1000 ml:

| | | |
|---|---|---|
| 62.5 | g | brewer`s yeast |
| 62.5 | g | cornmeal |
| 10 | g | agar-agar |
| 20 | g | glucose monohydrate |
| 30 | g | molasses |
| 30 | g | sugar beet syrup |

add 1000 ml $H_2O$
The ingredients were cooked for 10-15 min in the water bath, autoclaved for 15-20 min, and cooled down to 60 °C

Add

| | | |
|---|---|---|
| 10 | ml | propionic acid |
| 30 | ml | nipagin (in 70% Ethanol) |

fill directly into appropriate fly vials, storage at 4 °C after cooling

## 2.2 Methods

### 2.2.1 *Drosophila* culture and crosses

All flies were raised on standard fly food unless mentioned otherwise. Fly stocks were kept in *Drosophila* food vials with NM at 18 °C at a 12 h light/dark cycle. The flies for the experiments were taken from the stocks and kept at RT. The flies were transferred to a new medium every two to three weeks. The handling of the flies was performed on ice after anesthetization with nitrogen.

#### 2.2.1.1 *Drosophila* Stocks

The fly lines were obtained from the Bloomington *Drosophila* stock center, Indiana, USA (https://bdsc.indiana.edu/index.html), gifts from other research groups, or generated in our laboratory. Bloomington stock numbers and the name of the donor's research group are listed in materials Tab. 1.

#### *2.2.1.2* The bipartite GAL4/UAS-Expression system in *Drosophila*

Targeting gene expression temporally and spatially has proven to be one of the most powerful techniques for addressing gene function *in vivo*[146]. The Gal4/UAS-expression system in *Drosophila* allows the study of ectopic expression of genes in different spatial and temporal expression patterns. The Gal4-UAS system is originally derived from the yeast *Saccharomyces cerevisiae*. The GAL gene family is required for galactose metabolism in yeast and comprises structural (GAL1, GAL10, GAL2, and GAL7) and regulatory (GAL4, GAL80, and GAL3) genes. The transcriptional regulatory protein, GAL4, binds to 17 base pair sites referred to as the Upstream Activating Sequences (UAS) to activate the GAL10 and GAL1 target genes. The UAS is analogous to an enhancer element defined in multicellular eukaryotes[147] and is activated in the yeast by the presence of galactose.

In this system, the expression of the gene of interest, the responder, is controlled by the presence of the UAS element. Because transcription of the responder requires the presence of GAL4, the absence of GAL4 in the responder lines maintains them in a transcriptionally silent state. To activate their transcription, responder lines are mated to flies expressing GAL4 in a particular pattern, termed the driver (Fig. 7). The resulting progeny then express the responder

in a transcriptional pattern that reflects the GAL4 pattern of the respective driver. For example, by fusing a gene encoding a visible marker such as GFP (Green Fluorescent Protein), the expression pattern of the driver genes can be determined, as depicted in Fig.7.

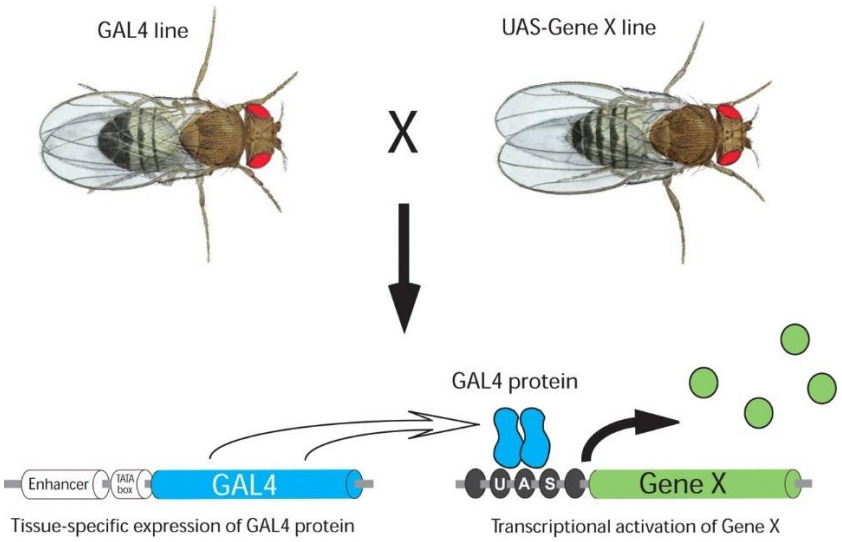



**Figure 7. The bipartite UAS/GAL4 system in *Drosophila***. When females carrying a UAS responder (UAS--Gene X) are mated to males having a GAL4 driver, progeny containing both elements of the system is produced. The presence of GAL4 in specific tissue or cells in the embryos then drives the expression of the UAS responder gene in a corresponding pattern [148].

### 2.2.1.3 General crossings

Crossings were performed using the Gal4/UAS system to get the desired genotypes. The virgin females (5-15) were collected and mated with the corresponding males in a 1:3 ratio. All experiments were additionally performed with the Gal4/UAS lines crossed to $w^{1118}$ as control. The crossings were kept at 25 °C, in constant, controlled light/dark cycles and humidity, unless mentioned otherwise. The parental flies were put on a new food vial at least every four days. All experiments were performed with the L3 larvae of the F1 generation.

### 2.2.1.4 Thermogenetic Activation of dTRPA1 in dIlp2 neurons

The GAL4-UAS system can be used for accurately manipulating neural activity. Dilp2-specific neuron clusters sufficient to induce insulin signaling were triggered by activating a UAS for the thermosensitive $Ca^{2+}$ -permeable cation channel, TrpA1 (UAS-TrpA1), that depolarizes neurons when flies are exposed to a temperature above 25°C. At 15–20°C, axons expressing

dTRPA1 do not generate action potentials; however, once the surrounding temperature reaches above 25°C, dTRPA1 expressing axons fire spikes tonically [149][150]. Pairing UAS-TrpA1 with *dIlp2*-GAL4 flies induces insulin signaling at temperatures above 25°C. dTRPA1 activation of dIlp2 secreting in the neurons of *dIlp2*-GAL4 x UAS-*dTRPA1* animals can be achieved using a simple heating protocol. To activate dTRPA1 channels in the brain of F1 larvae for achieving persistent, low-level activation of the *Ilp2* neurons throughout larval life by expressing channel TrpA1 from *Ilp2-GAL4* in larvae reared at 25°C. Control setup was maintained at 18°C. Figure 7A shows a schematic of dTRPA1channel activation by heat. Temperature-treated *dIlp2*-GAL4 x UAS-*dTRPA1* larvae were examined for branching phenotype.

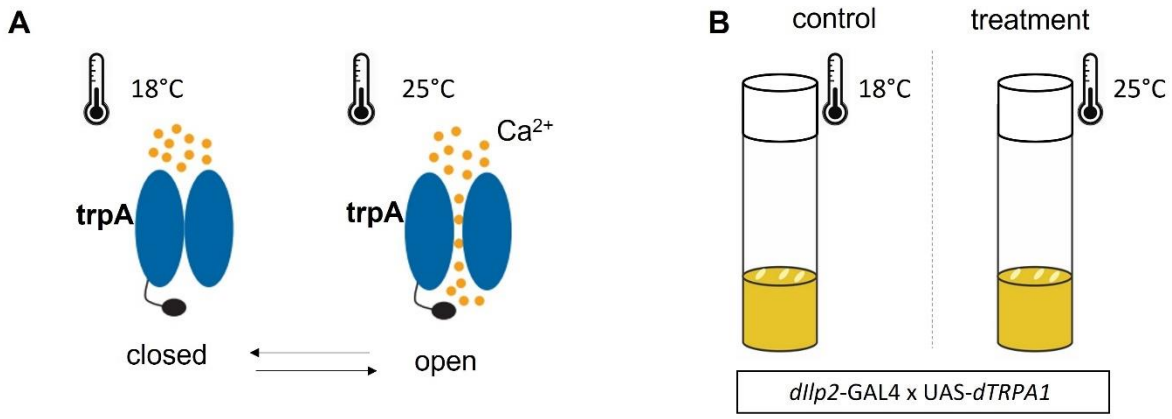



**Figure 8. TRPA1 channel is activated at temperatures from 25°C in *Drosophila* larval neurons** A) Neurons expressing *Drosophila* TRPA1 (dTRPA1) fire tonically in response to modest heat pulses. B) dTRPA1 expression is limited to neurons (dILP2) alone (dILP2-GAL4 x UAS-dTRPA1).

### 2.2.1.5 Optogenetic Activation of the ILPs secreting dIlP neurons with blue light

Binary expression systems, combined with optogenetics, can be used to control neuronal function in selectively small populations of neurons or even individual cells. Optogenetics is a technique adapted to control neuronal activity non-invasively and with high spatial and temporal specificity. This method is based on the genetic expression of light-gated ion channels in the cell membrane, enabling activation and inhibition of neurons in response to light. Channelrhodopsin-2 (ChR2) is a microbial-type rhodopsin that can be genetically expressed under UAS control which allows the depolarization of cells by blue light (480 nm) [151]. ChR2 absorbs blue light, resulting in its conformation change from all-trans retinal to 13-cis retinal that opens the channels (Fig.7). Consequently, ion influx happens across the plasma membrane

through ChR2, resulting in neuronal activity. A mutant (D156C) form of ChR2, named ChR2-XXL, is a potent tool for controlling neural activity. It displays increased light sensitivity, extra high expression, and extended open-state resulting in larger photocurrents[152].

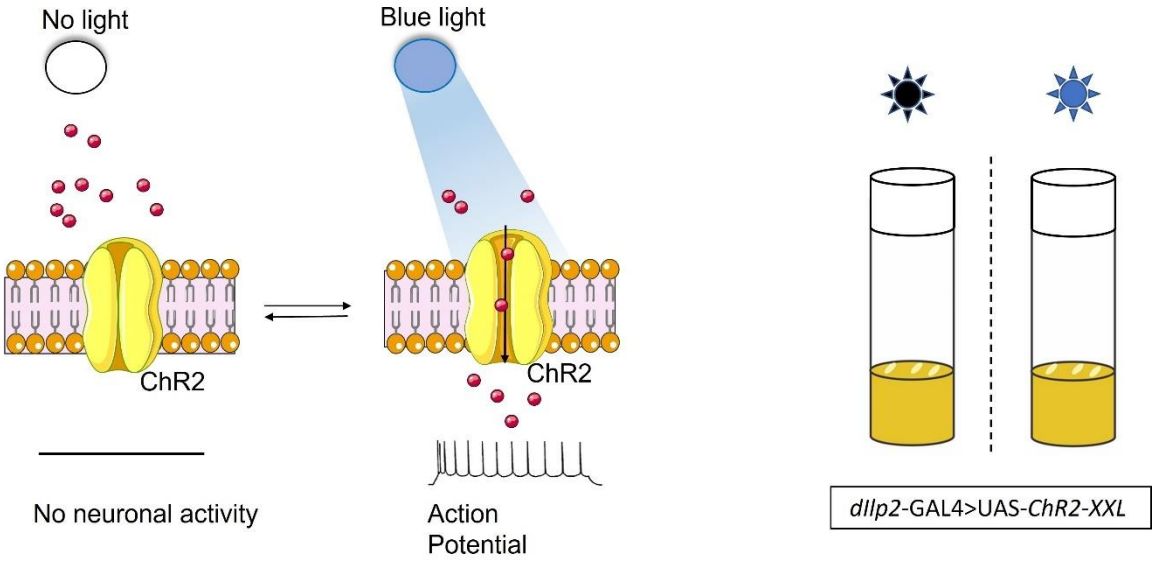



**Figure 9. Light-induced opening of channelrhodopsin allows ions to flow into the neuron, resulting in neuronal activation.** Channelrhodopsins conduct cations**,** depolarize neurons upon illumination to the light of a specific wavelength, and initiate individual action potentials (right). An unilluminated channelrhodopsin-2 (ChR2) ion channel is closed and inactive(left). (B) Once exposed to the light of a specific wavelength(∼ 737 lux), ChR2 allows certain positive ions into the cell, activating ChR2 specifically in the dILP2 neurons of the larval brain, redrawn from [153].

To investigate branching phenotype under induced insulin signaling in the brain of *Drosophila* larvae, Dilp2 specific Gal4-driver paired with UAS- ChR2-XXL was used. Transgenic late L2 instar larvae expressing the ion-channel ChR2-XXL were subjected to blue light and raised until L3. The blue light lamp was arranged approximately 25 cm above the vials, and a lux meter was used for measuring illumination (≃ 737 lux). The same crossings that were covered with aluminum foil which were light proofed, were used as controls. The controls were also treated in the same way. The F1-generation of both control and treatment crosses were covered with aluminum foil to protect them from light until the start of blue light exposure. L3 larvae were prepared for microscopy and observed for branching phenotype.

### 2.2.2 Hypoxia treatment

Third instar larvae were collected from each genotype and placed in separate vials containing standard *Drosophila* medium for each hypoxia experiment. Three replicate populations were used for each genotype. After counting the larvae and transferring them to fly vials with media,

they were given a 4-hour recovery period before moving to the exicator (Fig.8). The vials with larvae were sealed with larvae parafilm. The surface of the parafilm was punched with small holes to ensure gas exchange. Hypoxia exposure was done by fine regulation of $N_2$ pumped into the chamber through the nozzle on the side until the oxygen electrode showed a 5% $O_2$. The excicator was sealed to ensure that air exchange didn't happen. $O_2$ concentration in the chamber was constantly measured through the $O_2$ electrode connected to the exicator. The humidity was maintained with a small bowl of water inside the chamber to prevent animals from drying out. The vials for each respective genotype were placed in an identical excicator containing normal atmospheric oxygen concentrations (normoxia). The flies were subjected to hypoxia or normoxia for the time period mentioned in each experiment. For scoring the branches, early L2 larvae were subjected to 5% hypoxia for three days, and the branches were scored from the same larvae after they reached the L3 stage.

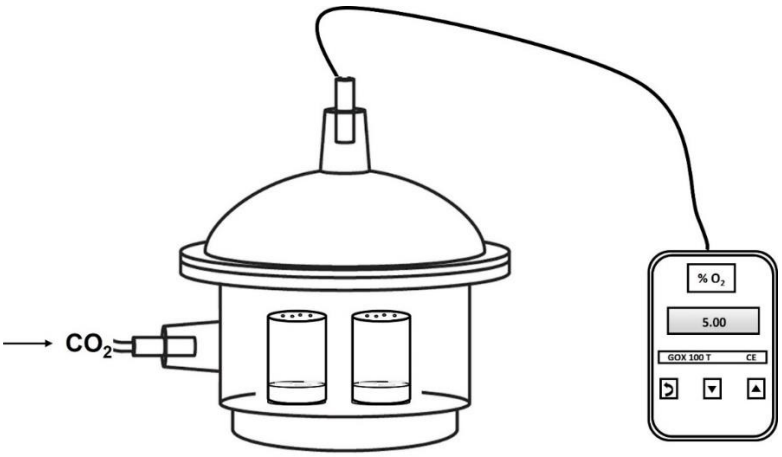



**Figure 10. Experimental design for application of hypoxia on *D. melanogaster* larvae.** . Hypoxia experiments were carried out using an airtight glass exicator supplied with $N_2$ gas. An oxygen electrode was used to measure the $O_2$ level within the exicator until the electrode showed a required reading of $O_2$ %. Humidity was maintained by passing the gas over water. Standard treatment condition was used during the whole experiment.

### 2.2.3 Dissection of trachea from L3 larvae

For the immunohistochemistry procedures, the required tissue(trachea) was dissected. For all experimental setups, L3 larvae were used. The larvae were washed in PBS to get rid of the remaining food medium

#### 2.2.3.1 Immunohistochemistry and Microscopy

Third instar larvae (early 3rd instar larvae) were dissected inside-out in PBS with 4% paraformaldehyde and fixed for 20 minutes. The trachea was washed six times 10 minutes with 0.1% Triton X-100 and 10% NGS in PBS, and then incubated with the primary antibody overnight. After washing six times for 10 minutes with 0.1% Triton X-100 in PBS, the secondary antibody was applied for 2 h. All antibodies were diluted in the respective washing buffer. In all tissue samples, nuclei were stained with diamidinophenylindole (DAPI) and mounted with 80% glycerol in PBS. Anti-dFoxO antibodies were diluted 1:1000 (Cosmo Bio Co., LTD) and were used to visualize the margin of the epithelial cells of the trachea. Microscopic analysis was performed either with an Olympus SZX12 stereomicroscope equipped with epifluorescence support or with a Zeiss Axio Imager Z.1 microscope.

### 2.2.4 LacZ staining /β-galactosidase staining

The trachea of the L3 instar larva was isolated in 1xPBS and incubated for at least 25 min in the fixation solution. After fixation, the tissues were rinsed two times for 5 min with 1xPBS. The tissues were immediately covered with pre-warmed, freshly prepared X-Gal staining solution and were incubated at 37° C for 16 hours. The X-gal solution was removed, and the tissues were three times each in PBS. The tissues were then mounted in glycerin, and the slides were stored in the dark at 4° C to preserve staining intensity. Pictures were taken using the SZX12 microscope (Olympus, Hamburg, Germany) under identical conditions for both samples. For analytical purposes, control and hypoxia treated animals were treated the same way.

### 2.2.5 BODIPY staining

The neutral lipids in the whole body were stained with the amine-reactive 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY® 493/503) dye, which could be used to create green fluorescent bioconjugates. The dye was dissolved in dimethyl sulfoxide (DMSO, 1mg/ml) and diluted 1:1000 (v/v) in PBS for a working solution. For each genotype and experiment condition, larvae were dissected in HL3 solution and washed twice with cold 1x PBS. After washing, the PBS buffer was removed entirely, and 500 µl 4% (w/v) paraformaldehyde (PFA) was added for fixation for 15 min at RT. Subsequently, the fat body tissue was washed again with 1x PBS. Next, 200 µl of BODIPY dye was added over the fat

tissue and incubated for 1 h in the darkness at RT. Two more washes followed in PBS, and the preparations were observed under the fluorescent microscope.

### 2.2.6 Measuring cell fluorescence using ImageJ

ImageJ was used to Determine the level of cellular fluorescence from fluorescence microscopy images of TTCs expressing FoxO-GFP. Scale from the microscopy image was fed to ImageJ. The cell of interest was selected using any drawing/selection tools (freeform). The area integrated intensity and mean grey value was determined and measured using "set measurements" in the analyze menu. A region next to the chosen cell with no fluorescence was selected and treated as the background. The same area integrated intensity and mean grey value was determined and measured for the background. This step was repeated for the other cells whose fluorescence had to be measured.

The following formula was used to calculate the corrected total cell fluorescence (CTCF).

*CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings)*

### 2.2.7 Visualization and Scoring of Tracheal Growth

For visualization of tracheae using DIC optics, larval tissues were mounted in 100% glycerol and were immediately used to quantify/image phenotypes using a Zeiss Axioplan equipped with a Leica DFC425C camera. Third-instar larvae were heat fixed by heating them shortly at 70°C for a few seconds, and ramifications of the third dorsal branch (Fig.9) were counted under a bright-field microscope for both larvae under hypoxia and normoxia [88]. The ramification was measured for the third instar larvae after rearing the embryos on respective nutritional regimes for the nutrition-dependent branching experiment. Tracheal coverage was measured in specific regions of the body wall and was scored in mid-3rd–instar larvae as previously done by Centanin *et al*. [88]. In the cases where it was necessary to correct for developmental delay (severe nutrient restriction), the developmental delay was first monitored and found to be ca. 24 hr at 72 hr AEL. Egg collections and transfers to fresh plates were thus carried out 24 hr earlier to obtain 3rd-instar larvae at the same time as controls. Mutants and controls were mounted and processed on the same slides when comparing genotypes. The anterior to the top and the right dorsal terminal branch is always shown in body wall images.

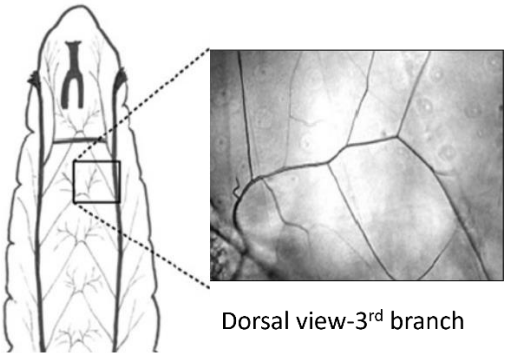



**Figure 11. Schematic View of the third segment of the tracheal branching on the dorsal side of larvae.** A) Scheme of a dorsal view of a *Drosophila* third-instar larva showing the position of a third segment dorsal branch terminal cell. B) Photograph shows the scheme of the morphology of a terminal cell of a wild-type larva grown in normoxic conditions. A typical dorsal branch terminal cell at this position comprises one main cellular process from which five or sometimes six main cellular extensions of more than one μm diameter are seen. Adapted from [88].

### 2.2.8 Measurement of Hypoxic Growth Restriction

All the eggs were collected 4 hours after egg laying to synchronize the age and then were maintained at normoxic conditions at 25°C for 48hrs. The L2 larvae were then subjected to hypoxia by maintaining them at 3.5% $O_2$ at 25°C. Controls were maintained at normoxic conditions. Larval growth assessment was done at 96 hAEL by immobilizing on ice and obtaining light micrographs. Lengthwise measurements were taken for genotypes DSRF>$w^{1118}$ and DSRF>FoxO-RNAi treated under normoxia and hypoxia. The length of the larvae was measured using the ImageJ software.

### 2.2.9 Visualisation of fat body opacity

Egg collection and hypoxia treatment for larvae were done the same way as mentioned above for Hypoxic Growth Measurement (2.2.6). Larval samples at 96 hAEL were immobilized on ice. The opacity of the fat body was visualized from images taken by light microscopy. Opacity was visualized for genotypes *dsrf*-GAL4 x $w^{1118}$ and dsrf-GAL4 x UAS-FoxO-RNAi treated under normoxia and hypoxia.

### 2.2.10 Nutritional Experiment

Our stocks usually are reared on a standard cornmeal/agar diet (6.5% yeast). For the experiments assessing the effect of yeast concentration on larval tracheal growth, the standard cornmeal/agar diet mentioned in materials (2.1.9) was used with the following three yeast

concentrations: 10%, 6.5% or 3.5%. “control” flies were raised and maintained in the nutritious cornmeal/agar diet with 6.5% yeast, whereas flies that were nutrient restricted as larvae were raised in the cornmeal/agar diet with 3.5% yeast, and then were switched to the nutritious cornmeal/agar diet with 10% yeast.

Flies of the different genotypes were reared on standard food and transferred to a more defined diet with differing nutritional content. Embryos were collected on different nutritional regimes and were let to grow until they reached the L3 stage. In the cases where it was necessary to correct for developmental delay (severe nutrient restriction or dFoxO KO), the developmental delay was first monitored and found to be ca. 24 hr at 72 hr AEL. Egg collections and transfers to fresh plates were thus carried out 24 hr earlier to obtain 3rd-instar larvae at the same time as controls.

### 2.2.11 TU-tagging: terminal cell–specific RNA isolation

Tu-Tagging method was used to label tracheal terminal cell specific RNA. The labelling and isolation protocol is described as below.

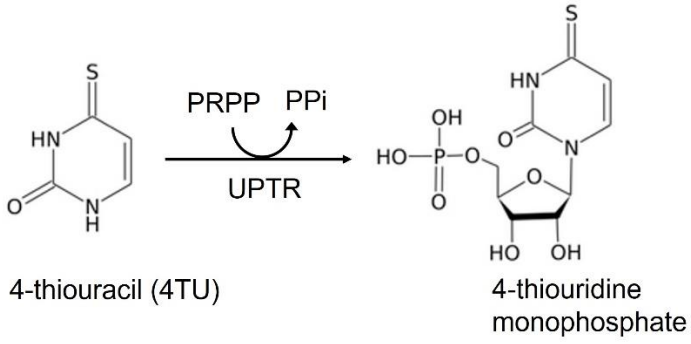



**Figure 12. Structures and inclusion chemistries of common RNA label-TU.**A) 4-thiouracil variants and pathways for incorporation into nucleotide metabolism; once the nucleotide monophosphate is formed, the resulting compound is readily incorporated into cellular RNA UPRT catalyses the conversion of uracil to uridine monophosphate in the presence of phosphoribosyl pyrophosphate (PRPP)

#### 2.2.11.1 Fly Crossings

Flies expressing UAS-HA: UPRT2.1 (chromosome II) were crossed with tracheal terminal cell-specific DSRF-GAL4 lines to express UPTR specifically in the TTCs. F1 larvae at the L3 stage were collected and used for TU treatment.

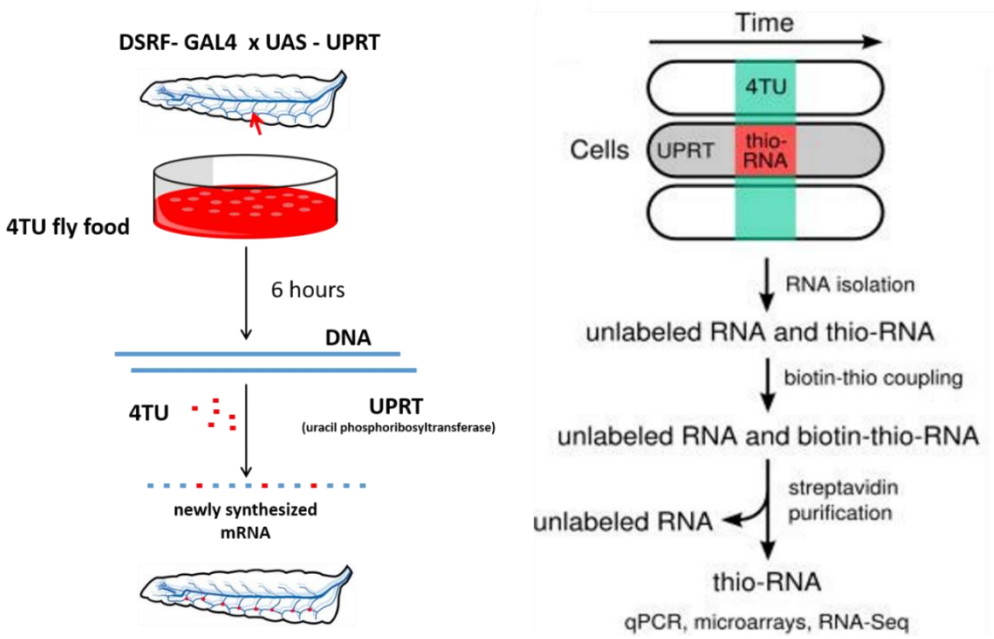



**Figure 13. TU labelling for isolating TTC specific RNAs.**:(A) UPRT catalyses the conversion of uracil to uridine monophosphate in the presence of phosphoribosyl pyrophosphate (PRPP). (B) A schematic of the 4TU-tagging technique proposed for cell-type specific labeling of RNA. (C) Workflow of 4TU labeling and detection of 4TU labeled RNA

#### 2.2.11.2 4TU treatment

To treat larvae with 4-thiouracil (4TU), the larvae were washed, and rinsed with sterile water. Blotted Larvae were transferred to fly food media containing 4-thiouracil (1.0 mM 4TU; Sigma- larvae of the desired stage were placed on food caps (20 ml $H_20$, 0.4 g sucrose, 0.18 g agar, 1 g dextrose, and 0.5 g brewer's yeast) containing 0.5 mM 4TU (Sigma-Aldrich 440736) for the indicated time at 30°C. After 6 hours, the larvae were moved using a paintbrush to a microcentrifuge tube with a screw cap, homogenized in 1 ml of Trizol, and stored at −80C until RNA purification.

#### 2.2.11.3 RNA isolation of TU-labeled samples

RNA isolation was performed using conventional RNA-magic isolation procedures. 30-50 animals per sample were used for the downstream isolation protocol. Larvae were transferred to 1ml RNA magic solution and immediately proceeded with tissue disruption using the Bead Ruptur protocol. LowBind 1.5 ml Tubes were used for extraction. After tissue disruption, 200 µl Chloroform was added and mixed well by multiple inversions. The mixture was incubated for a few minutes and centrifuged for 15 min (RT). The upper phase was transferred to a new tube leaving out the interphase. 1 ml of isopropanol was added to the separated upper phase.

The content was centrifuged for 20 min (4°C). The pellet was washed twice in 75% Ethanol, dried and resuspended in A. dest. Only RNA samples with A260/280 ratios of ≥ 2.0 were used for subsequent biotinylation and purification steps. In all cases, RNA samples were resuspended at a final concentration of ≥ 0.4 µg/µl.

#### 2.2.11.4 Biotinylation of TU-tagged RNA

10µg of total RNA (in 100µl total volume) was used for Labeling. 10 µl 10X TE (made sterile and stored as aliquots; 100mM Tris/HCl pH 7.4, 10mM EDTA; pH 7.4) was added to the RNA. This was filled up with A. dest to 75µl volume. 25µl EZ-link-Biotin-HDPD (1mg/ml in Dimethylformamide; aliquots of 50µl can be stored at -20°C) was added as the last component. The components were mixed well and incubated for 1.5h at RT in the dark with agitation (rotation).

#### 2.2.11.5 Repurification of biotinylated mRNA

The RNA was purified (to eliminate unbound Biotin) using a conventional chloroform extraction protocol. The mixture was filled up to 500µl with 1X TE buffer. 1volume (500µl of Chloroform/Isoamyl alcohol (24:1) was added, mixed vigorously – incubated for 2 minutes, and then centrifuged all full speed for 5 min (RT) to induce phase separation. The upper phase was transferred into a new tube, and the procedure was repeated. Phase-lock tubes (2ml, Eppendorf) were used for the second round.

#### 2.2.11.6 RNA precipitation

1/10 volume of 5M NaCl was added to the mixture and mixed well. One volume of Isopropanol was added, mixed well by inverting several times and centrifuged at max speed for 20 min (4°C). The supernatant was removed carefully, and the pellet was washed with 500 µl Ethanol (75%); centrifuge again for 10 min at max speed (RT). The supernatant was removed carefully; the pellet was dried and resuspended in 15 µl of A. dest (1µl for photometry). The protocol was proceeded with the isolation step.

#### 2.2.11.7 Isolation of biotinylated mRNA

The biotin-bound RNA was isolated with the labeled sample derived from the previous step. The RNA was directly used for streptavidin-bead mediated binding. 0.2 ml Tubes were used for this step. 25 µl of Beads were washed three times with binding buffer (1 M NaCl, 100 mM

Tris/HCl pH7.5, 10 mM EDTA, 0.1 % Tween 20) and resuspended in 25 µl binding buffer. The RNA from the previous step was (e.g., resuspended in 30 µl A. dest, taken 25 µl, heated for 10 min to 65°C, and the beads in binding buffer were added to it (25 µl, pipette up and down several times to mix). The mixture was incubated at RT for 15 min in the dark. The beads were washed three times with washing buffer (100 µl each) at RT and once with prewarmed buffer at 65°C. This sample was directly used for the cDNA synthesis on the beads.

#### 2.2.11.8 cDNA-synthesis of biotinylated mRNA

The cDNA synthesis was performed directly on the beads without isolating the labeled RNA.

Preparation of complementary DNA (cDNA)

cDNA is a DNA copy synthesized from mRNA. The enzyme reverse transcriptase is an RNA-dependent DNA polymerase isolated from a retrovirus (AMV or MMLV). As with other polymerases, a short double-stranded sequence is needed at the 3' end of the mRNA, which acts as a starting point for the polymerase. This is provided by the poly (A) tail found at the 3' end of most eukaryotic mRNAs, to which a short complementary synthetic oligonucleotide (oligo dT primer) is hybridized (polyT-polyA hybrid). Together with all 4 deoxynucleotide triphosphates, magnesium ions and at neutral pH, the reverse transcriptase synthesizes a complementary DNA on the mRNA template. The protocol is put into practice as follows, 400ng of total RNA was added in a 200 µl sterile tube on ice, mixed gently with 1 µl Oligo (dT7 I), OligodT primer, dNTPs and filled up to a volume of 6.5 µl and spun down for 5 seconds in a microfuge. The mixture was incubated at 65 °C for 5 min, and the tube was placed on ice. The following components were added: 5 x reaction buffers, RibolockTM Ribonuclease inhibitor (20 U/µl) to prevent ribonuclease activity and DTT. The mixture was gently mixed and incubated at 37°C for 5 minutes. In the last step, Super Script IV RTase was added and set the mixture at 42 °C for 60 min. The reaction was stopped by heating at 70 °C for 10 min. The newly prepared cDNA could be used directly in PCR reaction or frozen in a –80°C freezer.

**Table 5. cDNA synthesis reaction mix**

| | |
|---|---|
| OligodT (38a) 10 pmol | 0.5 µl |
| dNTPs (2.5 mM each) | 0.5 µl |
| RNA (ideally 400 ng) | ? |
| $H_2O$ | ? (fill up with water to 6.5 µl) |
| Incubate for 5 min at 65°C (for denaturation of potential secondary structures). Incubate for 1 min on ice. | |
| 5 x SSIV buffer | 2 µl |
| DTT | 0.5 µl |
| RNase inhibitor | 0.5 µl |
| Super Script IV (reverse transcriptase) | 0.5 µl |

2.2.11.9 Polymerase chain reaction (PCR)

A polymerase chain reaction (PCR) was performed to amplify specific DNA segments. The selected, designed primers determine the region and size of the PCR product. The primers used were Rpl (internal control), DSRF (positive control for terminal cells), and DH44, OA2, and OAMB (brain-specific primers as negative controls). For PCR products with high importance of a sequence without any failures during PCR amplification the Pwo DNA Polymerase (Roche) with a 3`-5`exonuclease activity (proofreading activity) was mixed 1:1 with the Taq DNA polymerase.

The Taq DNA polymerase from Invitrogen was used after the manufacturer's instructions in a 25 µl reaction volume:

**Table 6. RT-PCR reaction mix and cycling conditions**

| | | |
|---|---|---|
| $H_2O$ | 18 µl | |
| 10 x buffer | 2.5 µl | |
| $MgCl_2$ | 1.5 µl | |
| dNTPs (2.5 mM each) | 0.5 µl | |
| Sense primer (10 µM) | 0.5 µl | |
| Antisense primer (10 µM) | 0.5 µl | |
| Taq polymerase | 0.2 µl | |
| Template | 1 µl | |
| | ~ 25 µl per reaction | |
| Cycling conditions: | | |
| 95 °C | 3:00 min | |
| 95°C | 0:30 min | x 35 |
| 55°C | 0:30 min | |
| 72°C | cycles | |
| 72°C | 0:30 min | |
| 4°C | 5:00 min | |
| | ∞ | |

2.2.11.10 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate DNA or RNA molecules by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate further than longer ones, thus separating them by size. To pour a gel, agarose powder was mixed with electrophoresis buffer (TBE) to the desired concentration (e.g., 1 % agarose solution), then heated in a microwave oven until it was completely melted (10 min, 400 W). After cooling the solution to about 60 °C, ethidium bromide was added to the gel. Then it was poured into a casting tray containing two combs, one in the middle of the gel and the other at one side, about 5 mm from the end, and allowed to solidify at room temperature or in a refrigerator. After the gel had solidified, these combs were removed, using care not to rip the bottom of the wells. The gel was still in its plastic tray, inserted horizontally into the electrophoresis chamber, and covered with TBE buffer until the gel, and the slots were coated. Samples containing DNA mixed with loading dye were then pipetted into the sample wells (30 µl), the lid and power leads were placed on the apparatus, and a current was applied (100 V, 400 mA). DNA migrates towards the positive electrode. When adequate migration occurred (usually after 30-35 min), DNA fragments were visualized using the gel documentation camera (Molecular Imager Gel Documentation, Bio-Rad, München).

1.5% agarose gel for gel electrophoresis:

| | | |
|---|---|---|
| 1.5 | % | (w/v) agarose |

Ad 100 ml with TBE, boil until it is clear, storage in a 60 °C water bath until use, add:

| | | |
|---|---|---|
| 4 | µl | (v/v) 1 % ethidium bromide |

An agarose gel electrophoresis was performed to prove the presence, quality, amount, and size of a PCR product. To determine the size of the separated nucleic acids, a DNA ladder was added to each gel. The electrophoresis was performed with 100 V for 30-45 min. Ethidium bromide intercalates into the DNA and is visible under UV light. The DNA bands were documented under UV light. All steps with ethidium bromide were performed with nitrile gloves.

### 2.2.12 Statistical analysis

All data were analyzed using student t-test or one-way ANOVA with R software for statistical computing and graphics(R-4.1.1). All graphs were illustrated using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). Results were expressed as group mean ± SEM. Differences in means were considered statistically significant when $p < 0.05$ unless otherwise stated.

### 2.2.13 Software

The following software were used for processing images and analyzing data.

*GraphPad Prism* 6.00 GraphPad Software, La Jolla, California, USA

*AxioVision 4.1*. Zeiss, Göttingen

*ImageJ* Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA

*R-4.1.1* R Foundation for Statistical Computing, Vienna, Austria

# 3 RESULTS

## 3.1 Oxygen and nutrition availability contribute to tracheal terminal cell plasticity in *Drosophila* larvae

In the larval stages of *Drosophila*, the tracheal system becomes plastic and adapts to the $O_2$ demand of different tissues of the body by ramifying and growing their terminal branches [98], [154]. It has been long established that there is a tight correlation between tracheal terminal branching and oxygen levels [154]. To visualize the branching of the TTCs, a terminal cell-

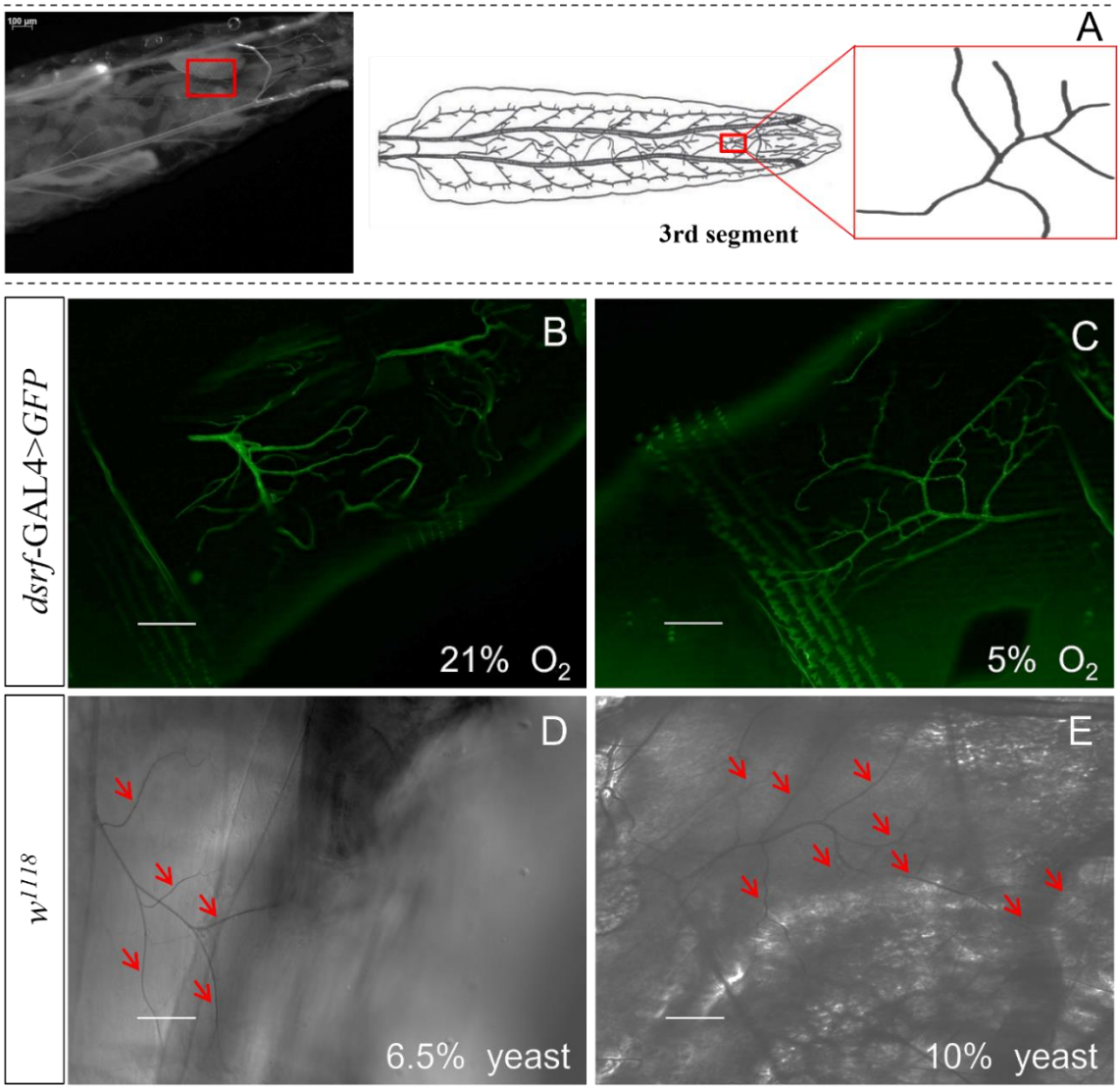



**Figure 14. Tracheal terminal cell plasticity in response to Oxygen and nutrient availability.** (A)Illustration of the third segment of the trachea. (B) An increase in the number of branches was observed in the TTC of *dsrf*-GAL4xUAS-GFP larvae subjected to hypoxia (5% $O_2$) compared to the control larvae(C) maintained under normoxia (21% $O_2$). The availability of an abundance in yeast nutrition (10% yeast) resulted in hyperbranching (red arrowheads mark branches of the TTC) in wildtype larvae(E) versus control (3.5% yeast) animals(D). Scale=50µm

specific driver, *dsrf*-Gal4 was used to achieve ectopic expression of GFP in the terminal cells. To demonstrate tracheal terminal cell plasticity to hypoxia, early L3 stage of *dsrf*-Gal4 x UAS-GFP larvae were collected and exposed to 5% $O_2$ in the oxygen chamber. As a control, a matching number of larvae were kept in 21% $O_2$ (normoxia). Fluorescence image of larvae subjected to hypoxia showed an increased number of branches and ramifications of the TTCs compared to their control kept under normoxia (Fig12.B&C, Fig.15). These results reinforce the previously proven tight correlation between tracheal terminal branching and oxygen availability. The results prove that the number of TTBs is a good read-out to analyze the extent of terminal branching in response to hypoxia [88].

Another recently recognized cue for terminal tracheal cell plasticity in *Drosophila* is nutritional abundance[100]. To validate TTC plasticity towards nutrition availability, wild-type larvae were reared on fly food with different yeast concentrations, and the branching phenotype was analyzed. Fly food with a 6.5% yeast concentration was considered a control, whereas fly food with a 10% yeast concentration was considered a nutrient-abundant medium. The terminal branches of the tracheae exhibited nutritional plasticity responding to abundance in yeast availability (10% yeast). They showed an increased number of terminal branches compared to control animals grown in normal food with 6.5% yeast concentration (Fig13.D&E, Fig.16). Collectively, these data support previously recognized plasticity of the insect tracheal system, driven by both nutritional and oxygen availability.

### 3.2 Hypoxia activates FoxO signaling in the airway epithelial cells

The transcription factor, FoxO, regulates various cellular processes, including the cell cycle, apoptosis, DNA repair, stress resistance, and metabolism. It is established that FoxO proteins are primarily retained in the cytoplasm when phosphorylated at the AKT sites. In contrast, FoxO, devoid of phosphorylation at the AKT sites, is translocated to the nucleus, subsequently activating FoxO signaling [155]. To study FoxO signaling under hypoxia, we ectopically expressed UAS-FoxO-GFP in the larval trachea, using the trachea-specific driver *pkk4*-Gal4, and subjected them to 5% $O_2$. Larvae subjected to hypoxia showed nearly complete nuclear translocation of FoxO in the airway epithelial cells of the dorsal trunk, primary and secondary branches, suggesting that the FoxO signaling is activated throughout the trachea due to hypoxia. This part of the research was recently published by our group[156].

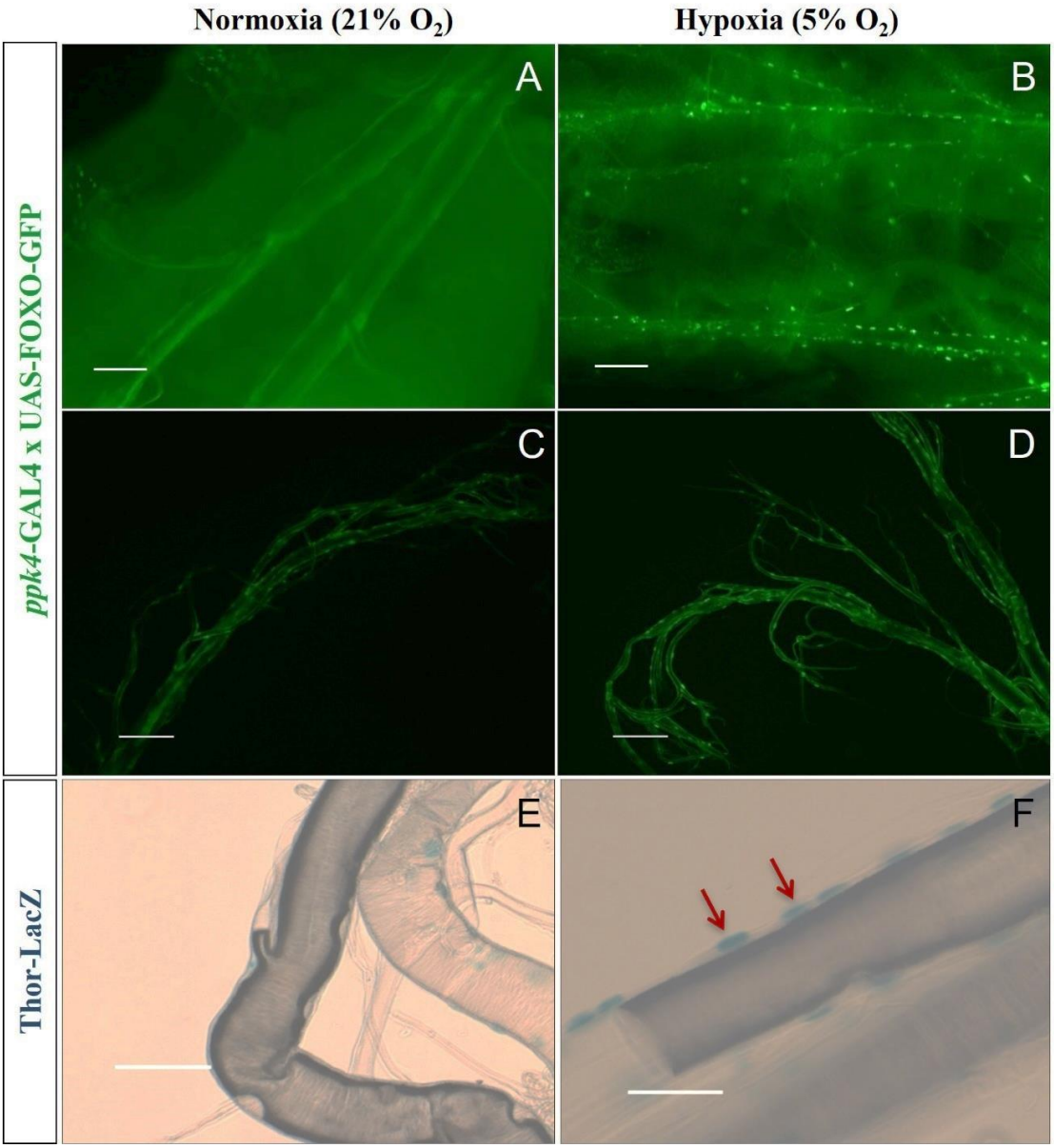



**Figure 15. dFoxO activation in the airway epithelium of larvae in response to hypoxia.** Fluorescence image showing nuclear translocation of *ppk4*-Gal4 driven dFoxO-GFP in the hypoxia-treated tracheal cells of the dorsal trunk in whole larvae(B) compared to the control(A)(scale=100µm). Nuclear translocation of dFoxO-GFP in the hypoxia-treated tracheal cells of dissected tracheal tissue. (scale=50 µm). X-Gal staining of dFoxO downstream target thor-LacZ reporter in control(D) and hypoxia treated cells(E). Red arrowheads mark X-Gal stained nuclei. Scale=50 µm

To further validate FoxO activation during hypoxia, we examined the expression of Thor throughout the trachea. Thor is a direct downstream target of FoxO; hence, Thor induction is a direct readout of FoxO activation [157]. The Thor(4e-bp) induction was examined by LacZ staining in *thor*-LacZ flies, a LacZ-enhancer trap in the *4e-bp* gene locus. When larvae were exposed to 2 hours of 5% $O_2$, they showed increased LacZ staining(blue) in the tracheal cell nuclei, indicating high expression of Thor under hypoxic conditions compared to control larvae under normoxia at 21% $O_2$ (Fig. 14E&F). X-Gal staining of Thor-LacZ further confirmed the

activation of FoxO signaling in the airway epithelium. Together, these data suggest that exposure to hypoxia in *Drosophila* larvae results in rapid induction of FoxO transcriptional activity throughout the trachea.

### 3.3 The mode of FoxO activity in the TTCs is different from the rest of the tracheal cells

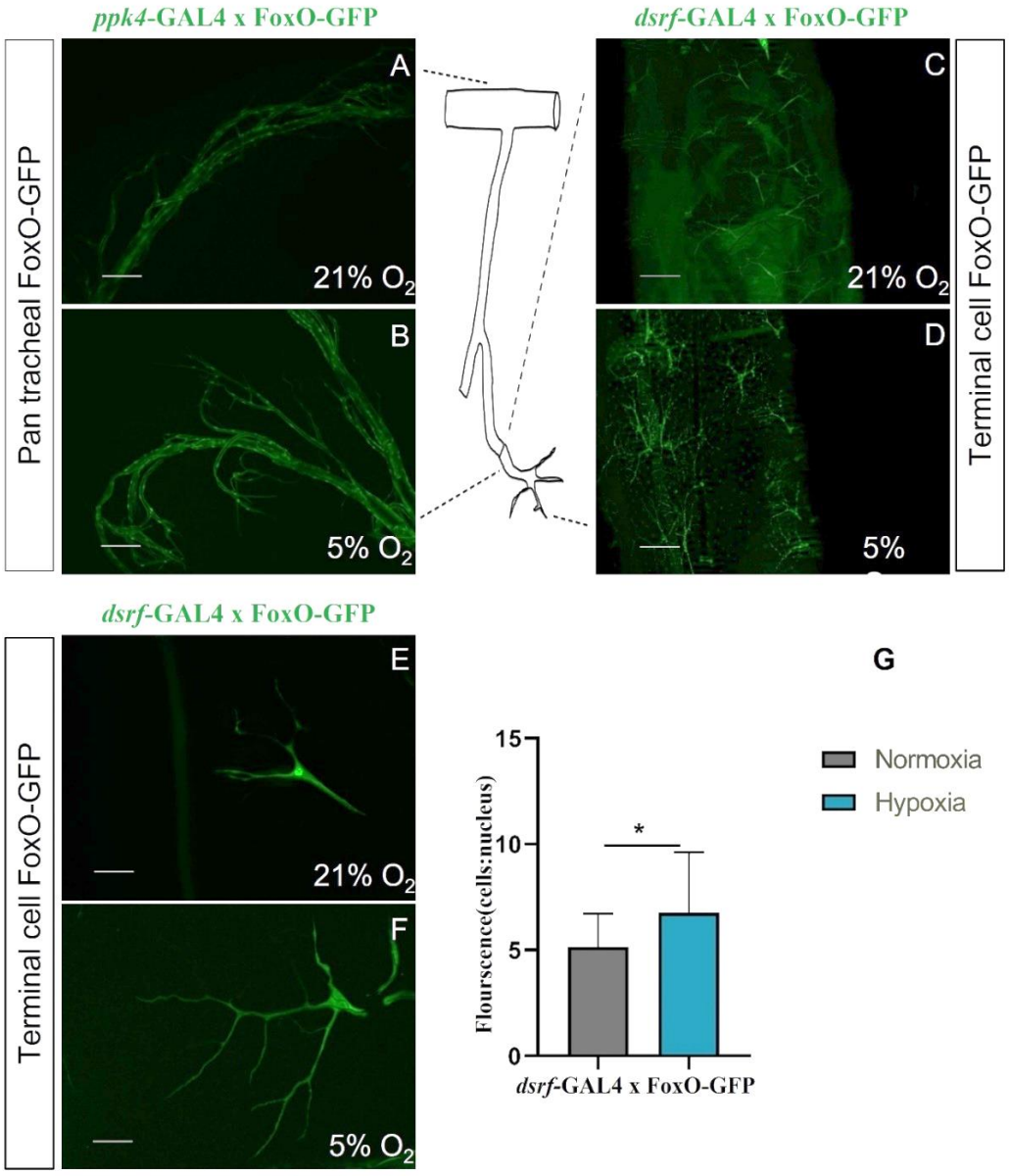



**Figure 16. FoxO signaling is activated in reverse by hypoxia in the tracheal terminal cells.** Hypoxia activated FoxO signaling throughout the trachea, and FoxO is translocated to the nucleus(B), whereas FoxO is retained in the cytoplasm in normoxia (A). Visualization of FoxO-GFP localized in the nucleus(C) and cytoplasm(D) of the terminal cells in normoxia and hypoxia, respectively. Scale = 200µm Magnified view of single terminal cells showing FoxO-GFP in normoxia (E) and hypoxia (F). Scale=50µm (G)The quantification of relative fluorescence emitted by dFoxO-GFP, obtained from the ratio of the cell body to the nucleus in the hypoxia-treated TTCs. Represented are means ± SD, $p<0.05$, students t-test. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

To visualize the activity for FoxO in the TTCs, we used dsrf-GAL, a driver specific for the TTCs, and ectopically expressed FoxO-GFP in the TTCs. Contrary to tracheal cells of the dorsal trunk, primary and secondary branches, FoxO in the terminal cells seemed to act in the nucleus under hypoxic conditions compared to the controls treated in normoxic states. To further quantify the amount of FoxO, the fluorescence emitted from the GFP expressed in the area of the nucleus and the cell body of the terminal cells was measured. The quantification of the fluorescent signals in the nucleus and cell body of the TTCs revealed an increased cytoplasm-to-nucleus signal ratio under hypoxia. The results suggest that the terminal cells respond to hypoxia via FoxO signaling differently from the rest of the tracheal cells. FoxO in the TTCs could be in its phosphorylated state, making it possible to be retained in the cytoplasm.

### 3.4 Hypoxia-induced terminal cell plasticity is mediated via FoxO signaling.

Given FoxO's ability to induce a different mode of sensitivity to hypoxia in the terminal cells, we hypothesized that perhaps FoxO activity inside the tracheal cells contributes to tracheal terminal sprouting in hypoxia. To investigate if FoxO regulates the terminal branch sprouting per se under hypoxia, we analyzed the branching phenotype of larvae with deregulated FoxO expression under hypoxia (5% $O_2$) and normoxia (21% $O_2$). We used larvae homozygous for *dfoxo* null alleles(*foxo*$^{21/21}$), larvae ectopically expressing FoxO in the terminal branch using a *dsrf*-Gal4 driver (*dsrf*-GAL4 x UAS-FoxO), and control (*dsrf*-GAL4 x w$^{1118}$) larvae under both normoxic and hypoxic conditions, to study the terminal cell branching phenotype. There was a reduction of the number of terminal branches in the *foxo* null larvae (3.50 ± 0.71) compared to the control (5.47 ± 1.17) in normoxic conditions. Ectopic expression of FoxO in the terminal cells also reduced the number of branches (3.33±1.44) in normoxia conditions. As expected, upon exposing these larvae to hypoxia, terminal branch sprouting was induced in the wild type (8.75±1.00). In FoxO knock out (4.56±1.01) and in larvae overexpressing FoxO (4.22±1.93), there was a severe reduction in the number and failed to show any induction of branching in hypoxia. These findings suggest that altered levels of FoxO protein distort the extra terminal sprouting phenotype observed in hypoxia; hence, FoxO activity inside the tracheal cells contributes to tracheal terminal sprouting in hypoxia.

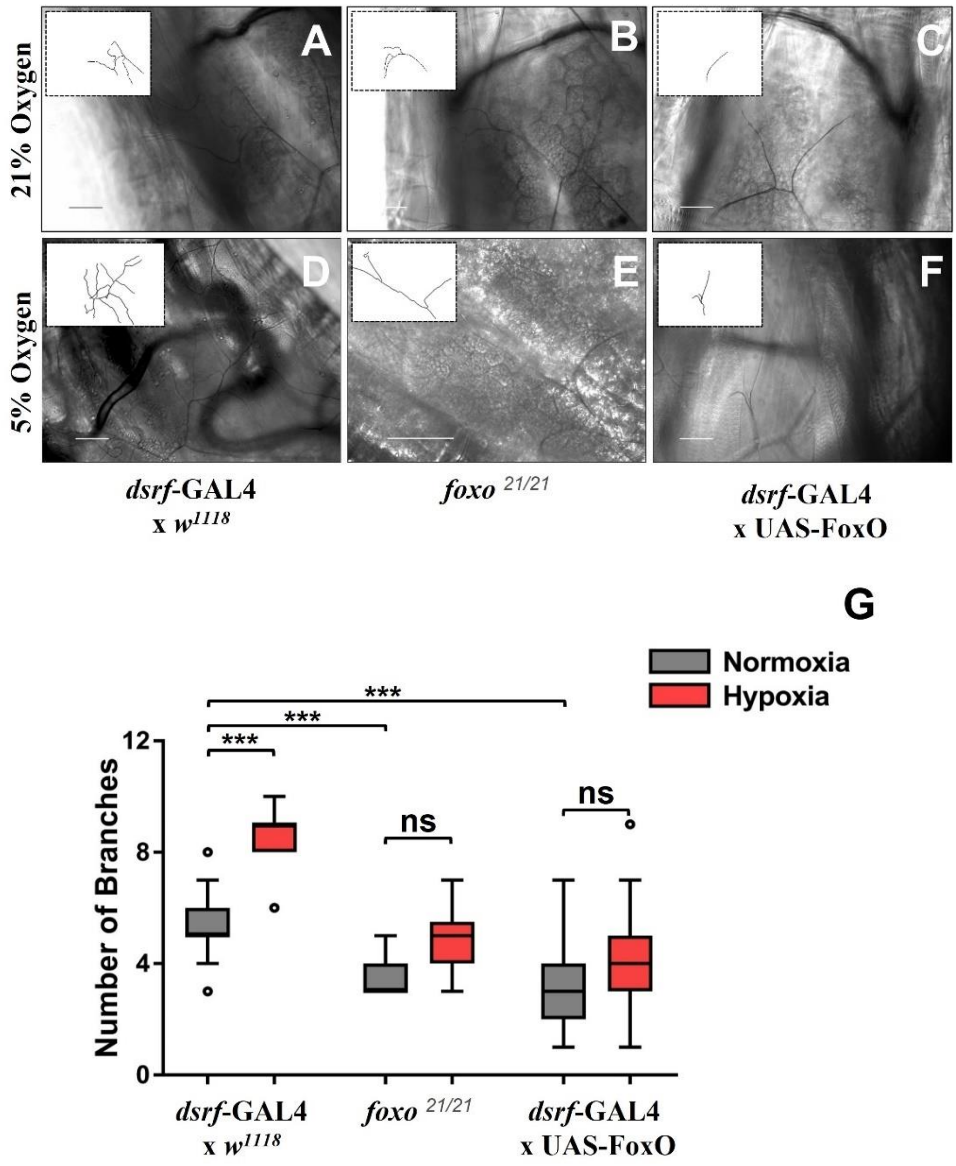



**Figure 17. FoxO regulates hypoxia induced branching in the tracheal terminal cells.** A-C) Representative tracheal branching of the third segment under normoxia control(A), foxo $^{-/-}$ (B), and dsrf-GAL4>foxo (C). D-E) Representative tracheal branching of the third segment under hypoxia in control(D), foxo $^{-/-}$ (E), and dsrf-GAL4> foxo (F). G) Quantification of branching in control, foxo $^{-/-}$ and dsrf-GAL4>foxo under normoxic and hypoxic conditions. The scale bar in all images is 50µm. n=10-24. Represented are means ± SD., p<0.001(ANOVA), Tukey's significance test, Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns = not significant

## 3.5 Terminal cell plasticity in response to nutrition is impaired upon deregulating FoxO signaling

Previous studies have demonstrated that tracheal terminal cells can exhibit nutritional plasticity by responding to previous and current dietary states[100]. To study if dFoxO has a role in regulating nutritional terminal cell plasticity, larvae homozygous for *foxo* null alleles(*foxo*$^{21/21}$), larvae ectopically expressing FoxO in the terminal branch (*dsrf*-GAL4 x UAS-*foxo*), and the controls (*dsrf*-GAL4 x w$^{1118}$) were grown in three different food regimes

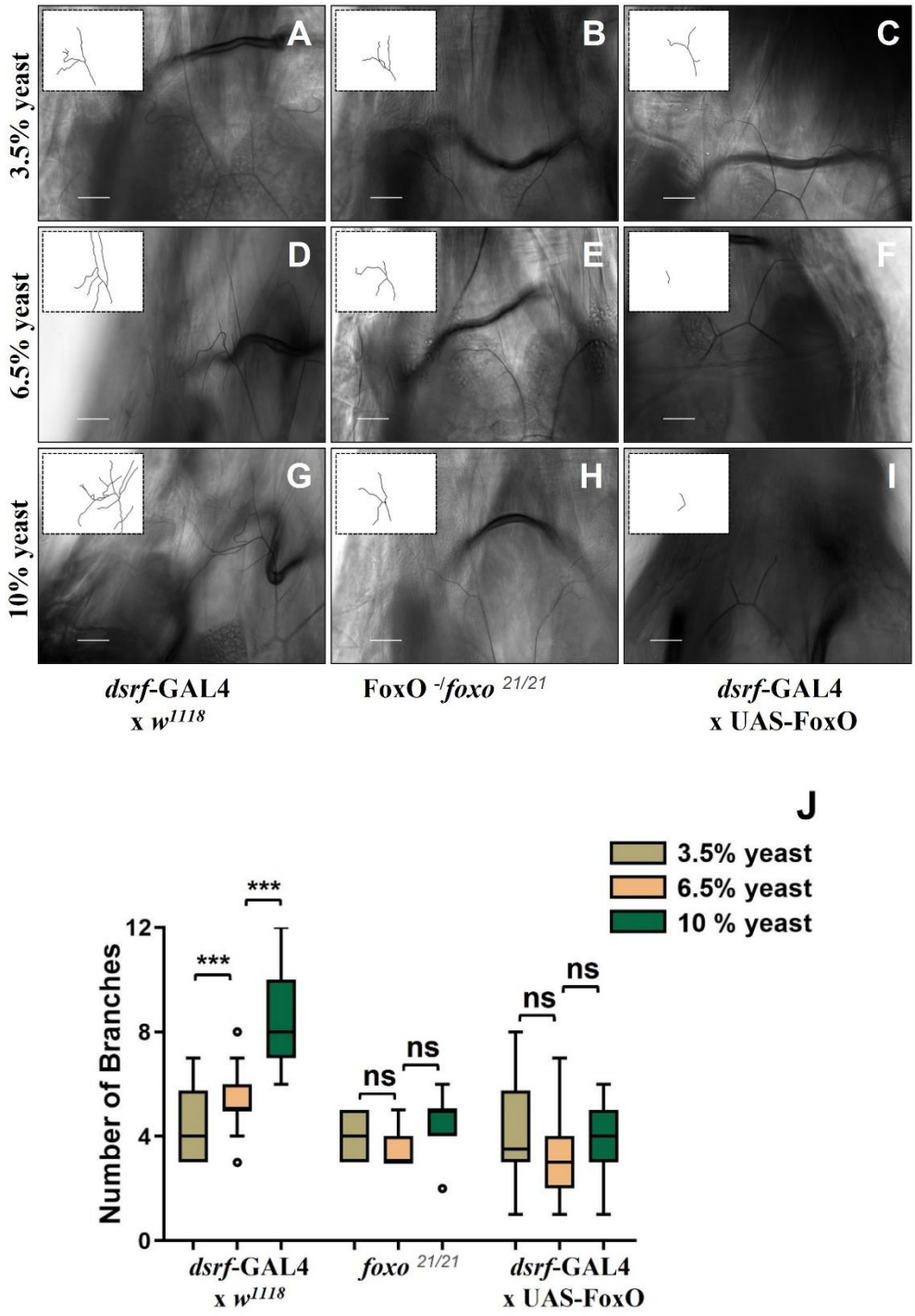



**Figure 18. FoxO regulates the nutrition-driven plasticity of the tracheal terminal cells.** A-C) Representative tracheal branching of the third segment in low (3.5% yeast) nutrition regime in control(A), foxo $^{-/-}$(B) and DSRF>foxo (C). D-F) Representative tracheal branching of the third segment under standard (6.5% yeast) nutrition regime in control(D), foxo $^{-/-}$ (E) and (F) DSRF>foxo (F). G-I) Representative tracheal branching of the third segment under a high (10% yeast) nutrition regime. J) Quantification of branching in control, foxo $^{-/-}$ and DSRF>foxo under various nutrition regimes. The scale bar in all images is 50µm. n=10-27. Represented are means ± SD., p<0.001(ANOVA), Tukey's significance test, Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns = not significant

with variable yeast content viz. 3.5% yeast (nutrition restriction), 6.5% yeast (control), and 10% yeast (nutrition abundance) and their branching phenotype were analyzed. An increase in dietary yeast led to increased tracheal coverage (8.33±1.56) and reduced dietary yeast showed a decrease in the number of tracheal branches (4.38±1.36) in the wild type compared to the control (5.47±1.21). FoxO-deficient animals on high yeast diet failed to show nutritional plasticity without any significant increase in the number of branches (4.65±0.75) compared to the animals grown in the control medium (3.50±0.71). *foxo*$^{21/21}$ on reduced yeast diet showed a branching of (4.06±0.89) was also not significant. Larvae with ectopically expressed FoxO in the terminal cells also failed to show plastic response in high yeast medium (3.89±1.44) compared to the control (3.83±1.24). The results clearly indicate that deregulating foxo in the terminal cells hinders the ability of TTCs to show nutritional plasticity.

### 3.6 Tracheal terminal cell exhibits increased plasticity on downregulating the expression of FoxO by RNAi

FoxO loss-of-function and FoxO overexpression in the terminal cells contributed to reduced tracheal branches and failed to exhibit plasticity under hypoxia. Similar results were obtained in response to nutritional abundance. Though these results indicate that FoxO has a role in regulating the branching of terminal cells, how the course of FoxO expression is the action of FoxO is not apparent from these results. To test if FoxO regulation is indeed the mechanism by which the terminal cell plasticity is regulated and to know the direction in which FoxO is acting, we silenced the FoxO in the terminal cells using an RNAi against the FoxO (*dsrf*-GAL4 x *foxo*-RNAi) in the terminal cells. Larvae with RNAi against the FoxO in the terminal cells showed an increased number of branches (8.55±1.53) than their control (5.9±0.91) under normoxic conditions. This pointed towards the fact that downregulating FoxO can lead to increased sprouting of terminal branches in contrast to the results obtained from FoxO deficient animals. These results indicate that excess FoxO and complete absence of FoxO disrupted the plasticity of the terminal cells, but FoxO at a low level promoted TTC plasticity. Thus, it could be assumed that FoxO expression in the TTCs has to be at a physiologically relevant level to induce plasticity of the terminal cells.

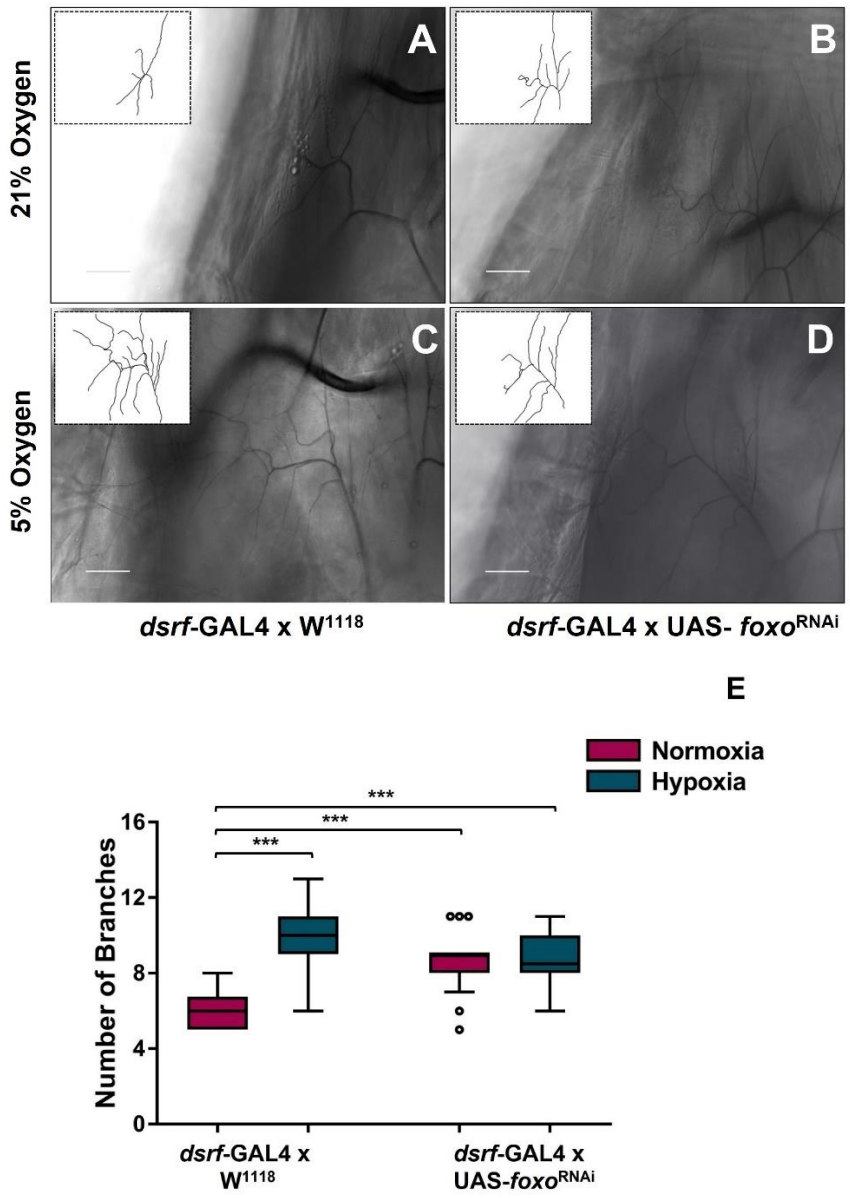



**Figure 19. Targeted reduced expression of FoxO in the terminal cell leads to hyper branching.** A-B) Representative tracheal branching of control(A) and *dsrf-driven* foxo-RNAi (B) under normoxia. C-D) Representative tracheal branching of control(C) and FoxO-RNAi(D) under hypoxia. E) Quantification of branching in control and DSRF>FoxO-RNAi under normoxia and hypoxia. The scale bar in all images is 50µm. n=21. Represented are means ± SD., p<0.001(ANOVA), Tukey's significance test, Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

### 3.7 Hypoxia-induced larval growth restriction can be lessened by FoxO regulation.

Like many other organisms, hypoxia restricts systemic growth and reduces body size in *Drosophila*. Limited oxygen has been linked to slow growth and developmental delay [158]–[160]. Rearing wild-type larvae under hypoxia reduced body size compared to larvae raised under normoxia. Since silencing *foxo* in the terminal cells increased the ramification of terminal branches even under normoxia, we next investigated whether this phenotype could

rescue the hypoxic growth restriction and whether downregulation of *foxo* could modulate organismal size.

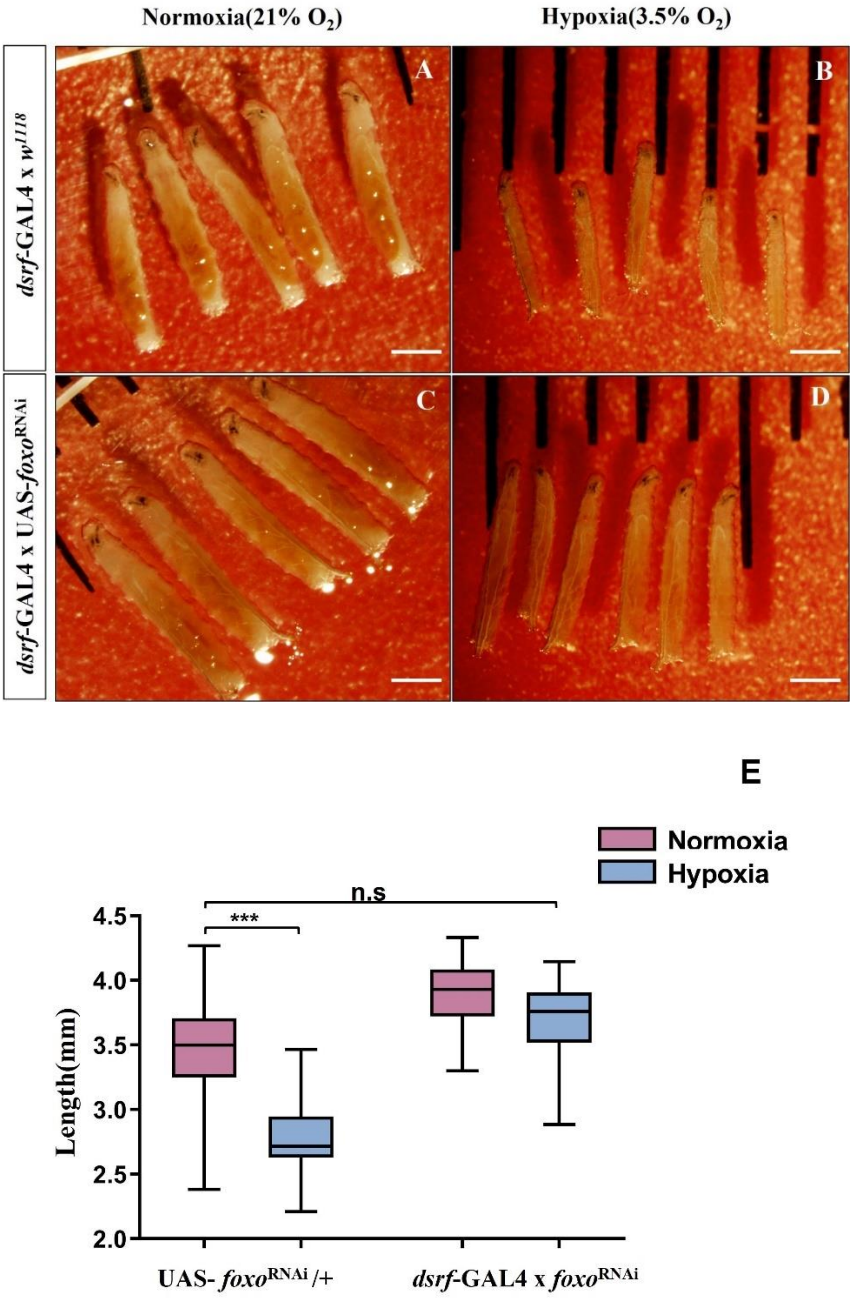



**Figure 20. Hypoxia restricts larval growth.** [A–B] Measurement of the length of wildtype larvae under hypoxic conditions resulted in a significant reduction in larval size (B) compared to those reared under normoxic conditions (A). [C–D] Larvae with downregulated *foxo* expression (*dsrf*-GAL4 x FoxO-RNAi) showed an increased length under hypoxia compared to its control under hypoxia. E) Quantification of length of control and *dsrf*-GAL4 x *foxo*-RNAi under normoxia and hypoxia Scale = 1mm. Represented are means ± SD. $p<0.001$(ANOVA), Tukey's significance test, Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns = not significant

Interestingly, the silencing of *foxo*, specifically in the larval TTC using *dsrf*-GAL4, mildly affected larval size (3.9±0.23 mm) with an increase in length under normoxic conditions (Fig.

20) compared to its control, *dsrf*-GAL4 x $w^{1118}$ (3.4±0.41mm). However, when larvae with foxo- RNAi were subjected to hypoxia, though there was a decrease in length (3,69±0.27mm) compared to its control in normoxia, the growth restriction was significantly reduced from the control larvae in hypoxia (2.7±0.26 mm). The results suggest that reducing FoxO in the terminal cells increases the terminal branching and might help the larvae cope better metabolically under hypoxic conditions.

### 3.8 Hypoxia induces lipid metabolism adaptations varied in larvae with FoxO downregulation in terminal cells

Larvae grown in hypoxic conditions typically exhibit decreased fat body opacity, which is comparable to what is observed in the nutrient-deprived fat body. As reported earlier, rearing wildtype larvae under hypoxic conditions showed characteristic decreased fat body opacity (Fig.21) compared to wildtype controls reared in normoxic conditions [102]. In normoxic conditions, larvae with FoxO-RNAi in the terminal cells (dsrf-GAL4 x UAS-foxo-RNAi) showed an opaque fat body, whereas when reared under hypoxic conditions, showed decreased opacity than its control (*dsrf*-GAL4 x w1118) in normoxia but was not as transparent as the wild type larvae raised under hypoxia. This observation demonstrates that the lipid immobilization that happens in the fat body during hypoxia is reduced to some extent by reducing the levels of FoxO in the TTCs, thereby rendering some opacity to the larvae.

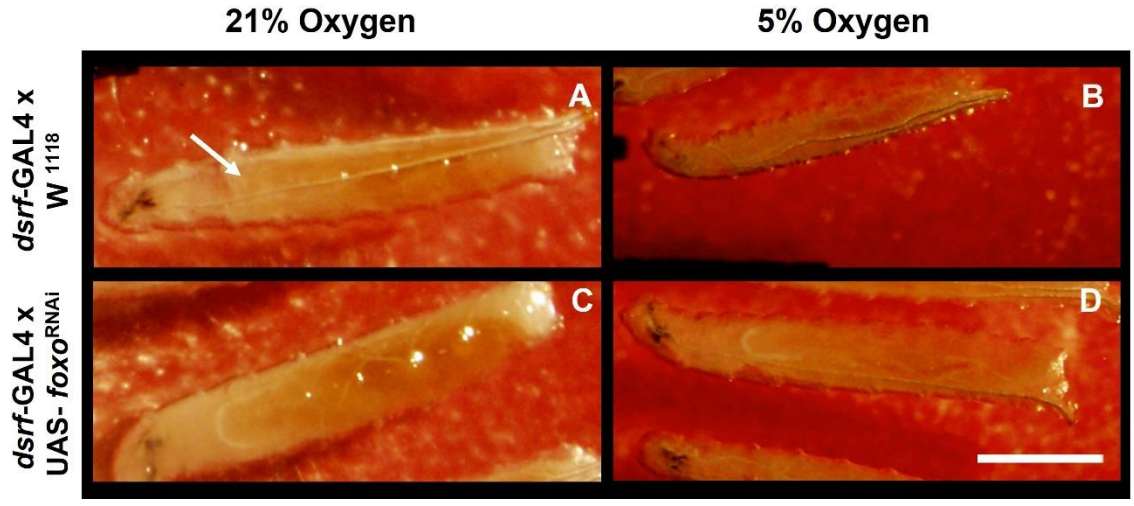



**Figure 21. Hypoxia decreases fat body opacity.** Control larvae showed decreased fat body opacity(B) when subjected to hypoxia, when compared to animals reared under normoxia (A). Larvae with reduced FoxO expression (*dsrf*-GAL4 x UAS-*foxo*-RNAi) under hypoxia showed an increase in fat body opacity(D), when compared to its control under hypoxia(B). White arrows point to the larval fat body. Scale=1mm

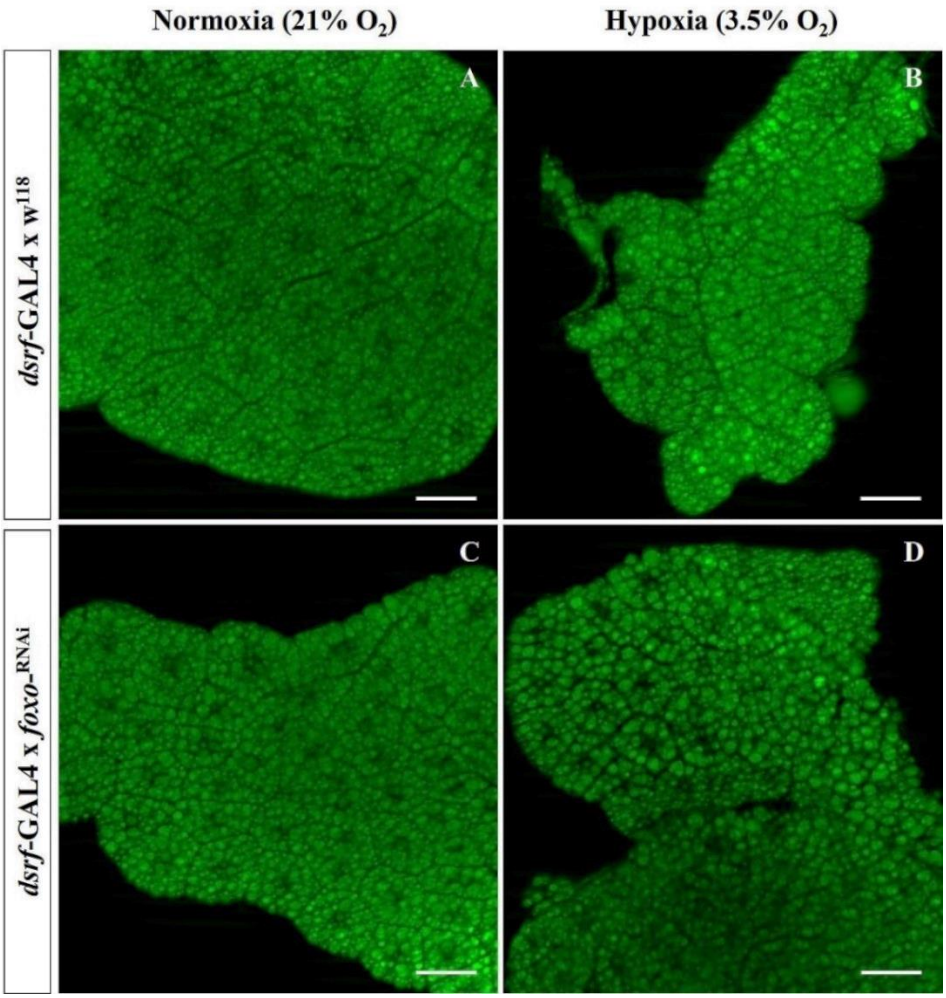



**Figure 22. BODIPY staining of the fat body showing lipid aggregation in tissue.** Control (*dsrf*-GAL4 x $w^{1118}$) larvae reared under hypoxic conditions exhibited increased lipid droplet accumulation in the larval fat body and loss of distinct cell boundaries (B) compared to the larvae raised under normoxia (A). *dsrf*-GAL4 x UAS-*foxo*-RNAi in hypoxia(D) showed a mix of large and small lipid droplets when compared to its control in normoxia (*dsrf*-GAL4 x $w^{1118}$)

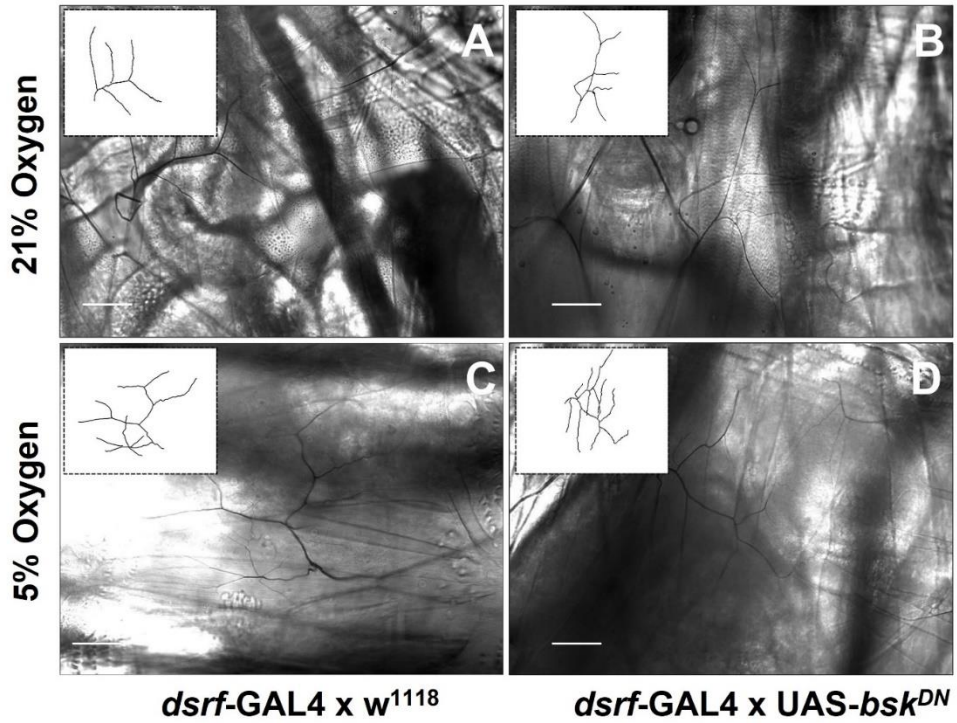
The neutral lipids in the fat body of larvae can be detected by staining with (BODIPY® 493/503) dye by forming a green, fluorescent bioconjugate. Bodipy staining of larval fat bodies showed that hypoxia caused lipids in this tissue to aggregate in larger droplets (Fig.20B), as compared to those observed in the wildtype control (Fig.22A). This increase in lipid droplet aggregation is relatable to that is observed during starvation [161], [162]. Hypoxia also causes the loss of distinct cell boundaries in the larval fat body. Larger lipid vesicle aggregation in the fat body indicates efflux of lipids into the hemolymph [162]; we investigated whether reducing FoxO in the terminal cells would influence the pattern of lipids mobilized from the fat body. However, when *dsrf*-GAL4 x UAS-*foxo*-RNAi larvae were subjected to hypoxia, the fat body showed large fat bodies. Still, the lipid droplets were less aggregated than wild-type larvae

under hypoxia. Also, the cell boundaries of cells in the fat body of *dsrf*-GAL4 x UAS-*foxo*-RNAi larvae in hypoxia were more distinct than its wild-type control in hypoxia. This might indicate that the lipid in the fat body of larvae with dFoxO downregulated in the TTCs is not readily immobilized or effluxed into the hemolymph as that of the wild-type larvae under hypoxia.

### 3.9 Disruption of JNK signaling in the TTCs did not influence the plasticity

Previous experiments from this study indicated that FoxO has a role in regulating TTC's plasticity. The nuclear/cytoplasmic shuttling mechanism is central to regulating FoxO factors, which enables FoxO factors to which excludes or includes them from exerting transcriptional activity. When cells are under stress, the JNK (c-Jun N-terminal kinase)-dependent signaling pathway causes phosphorylation of FoxO, thereby inducing FoxO nuclear translocation and transcriptional activity. Because JNK can activate FoxO signaling, we tested if the JNK pathway is required for FoxO activation to regulate the branching phenotype. To investigate this, the JNK signaling in the TTCs was suppressed by expressing a dominant-negative *basket* isoform, $bsk^{DN}$ (*bsk* is the; *Drosophila* c-Jun N-terminal kinase), using a terminal cell-specific driver, *dsrf*-GAL4. *dsrf*-GAL4 x UAS- $bsk^{DN}$ larvae were subjected to hypoxia (5% $O_2$). Matching number of larvae were kept under normoxia (21% $O_2$) was used control.

Terminal branch numbers of *dsrf*-GAL4 x UAS- $bsk^{DN}$ larvae, along with its control *dsrf*-GAL4 x $w^{1118}$ larvae were accounted and analysed to study the effect of supressed JNK activity in the tracheal terminal cells. However, on analysing the branching phenotype there was no significant change in the number of branches in larvae that expressed $bsk^{DN}$ (4.8±0.42) in the terminal cells, compared to its genetic control (5.4±1.72) under normal oxygen conditions (Fig 23. A, B). Even in hypoxic conditions, no significant change in the number of branches was observed in larvae expressing $bsk^{DN}$ (8.1±1.44) in the TTCs compared to its control (8.9±0.70), indicating that suppressing JNK activity in the TTCs did not affect its ability to sprout new branches under hypoxia (Fig 23. C, D). Thus, the TTC plasticity is not dependent on the segment of the signaling pathway comprising c-Jun N-terminal kinase (JNK) and FoxO signaling, most often activated in response to stress.



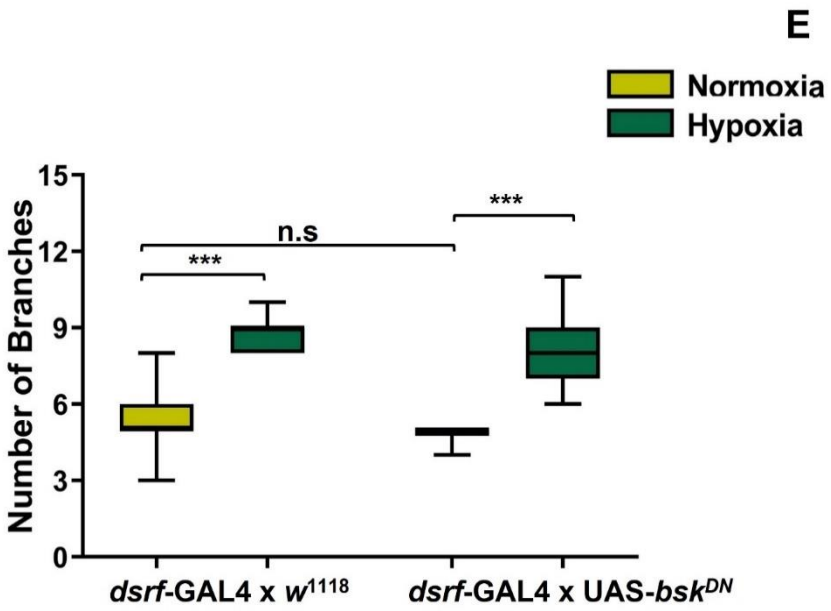




**Figure 23. Downregulating the JNK pathway in the terminal cells did not impact tracheal terminal cell plasticity.** Intervening with the JNK pathway using a dominant-negative basket isoform, $bsk^{DN}$ in the terminal cells did not affect the terminal cell's branch number under normoxia and did not affect its ability to sprout new branches under hypoxia. A-B) Representative tracheal branching of control(A) and DSRF driven UAS-, $bsk^{DN}$ (B) under normoxia. C-D) Representative tracheal branching of control(C) and dsrf-GAL4 x UAS-, $bsk^{DN}$ (D) under hypoxia. E) Quantification of branching in control and DSRF> UAS-$bsk^{DN}$ under normoxia and hypoxia. The scale bar in all images is 50µm. n=21. Represented are means ± SD., $p<0.001$(ANOVA), Tukey's significance test, Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns = not significant

## 3.10 Activation of Insulin signaling promotes tracheal terminal branching

As it was observed that FoxO activity was not dependent on JNK signaling and since FoxOs are effectors of the well-conserved insulin signaling pathway, we tested whether the FoxO-dependent hypoxia-induced terminal branch sprouting response is mediated by systemic *Drosophila* insulin-like peptides (dILPs) secreted from insulin-producing cells (IPCs) in the median neurosecretory cells (mNSCs). We hypothesized that larvae with increased dILP signaling should induce branching in the terminal cells as dILP acts upstream of FoxO. In the presence of insulin or insulin-like growth factors, the PI3K–Akt pathway is activated, which retains FoxO in the cytoplasm.

### 3.10.1 Optogenetic activation of Dilp2 neurons using ChR2-XXL

Light-gated ion channel channelrhodopsin-2 (ChR2) based optogenetic system was used to control the neuronal activity of the IPCs, and to induce secretion of dILPs from the muscles. When expressed in excitable nerve cells, ChRs can be used to control the membrane potential via illumination (blue light)[151]. Using a ChR2 mutant (D156C) called ChR2-XXL provides extra high expression and an extended open state and can be targeted to be expressed in the mNSCs using a *dilp2-specific* Gal4-driver[163]. Activation of ChR2-XXL expressed in the *dilp2* neurons enables these neurons to be electrically excited, resulting in the secretion of dILPs in the absence of a physiological stimuli.

ChR2-XXL under UAS control allows the depolarization of cells by blue light (480 nm). *dilp2*-GAL4 x UAS-*ChR2-XXL* larvae When the ion channel is activated with blue light). Before starting optogenetic experiments, animals were conditioned to darkness for approximately 10 min. Larvae were then stimulated with blue light (480 nm) or were continued under darkness(control). On activating the ChR2-XXL channels by applying blue light, terminal branching was induced (8.2±1.36) in the treatment group. However, there was an induction of branching in the control animals (7.3±0.86) kept under darkness, and the difference in the number of branches between the control and the blue light-treated larvae was not significant ($p=0.056$) (Fig. 24).

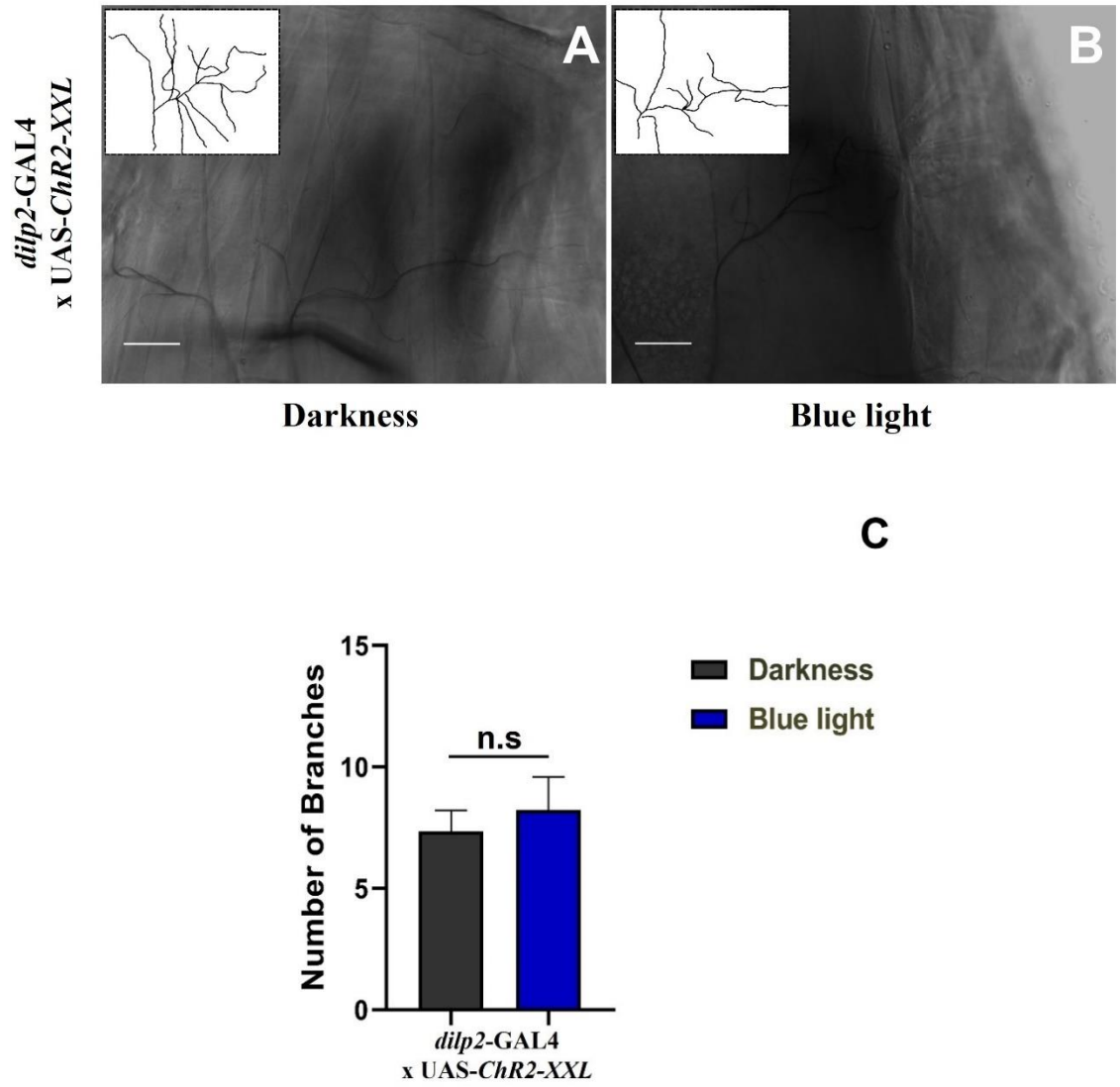



**Figure 24: Optogenetic activation of Dilp2 neurons using ChR2-XXL to induce increased insulin secretion IPCs.** A-B) Representative tracheal branching of dilp2-GAL4 x UAS-TrpA under control temperature and treatment temperature Quantification of branching dilp2-GAL4 x UAS-TrpA at control temperature and treatment temperature. The scale bar in all images is 50µm. n=15-16, Represented are means ± SD. $p<0.001$, Represented are means ± SD, $p<0.05$, students t-test. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05, ns = not significant

### 3.10.2 Thermogenetic activation of Dilp2 neurons using TrpA1

Since the above-described experiment that used ChR2-XXL to activate dilp2 neurons had shortcomings, a second experiment was designed that employed thermogenetic activation of dTrpA1 channels to induce dILP neurons. To induce dILP secretion in the IPCs, we used the *dilp2*-GAL4 driver[164] to manifest the expression of the temperature-sensitive TrpA1 channel (*dilp2*-GAL4 x UAS-*TrpA1*), which increases neuronal activity when larvae are

incubated at or above 25 °C[149]. The crosses were maintained at 25 °C to achieve a persistent low-level activation of the Ilp2 and the controls at 18 °C.

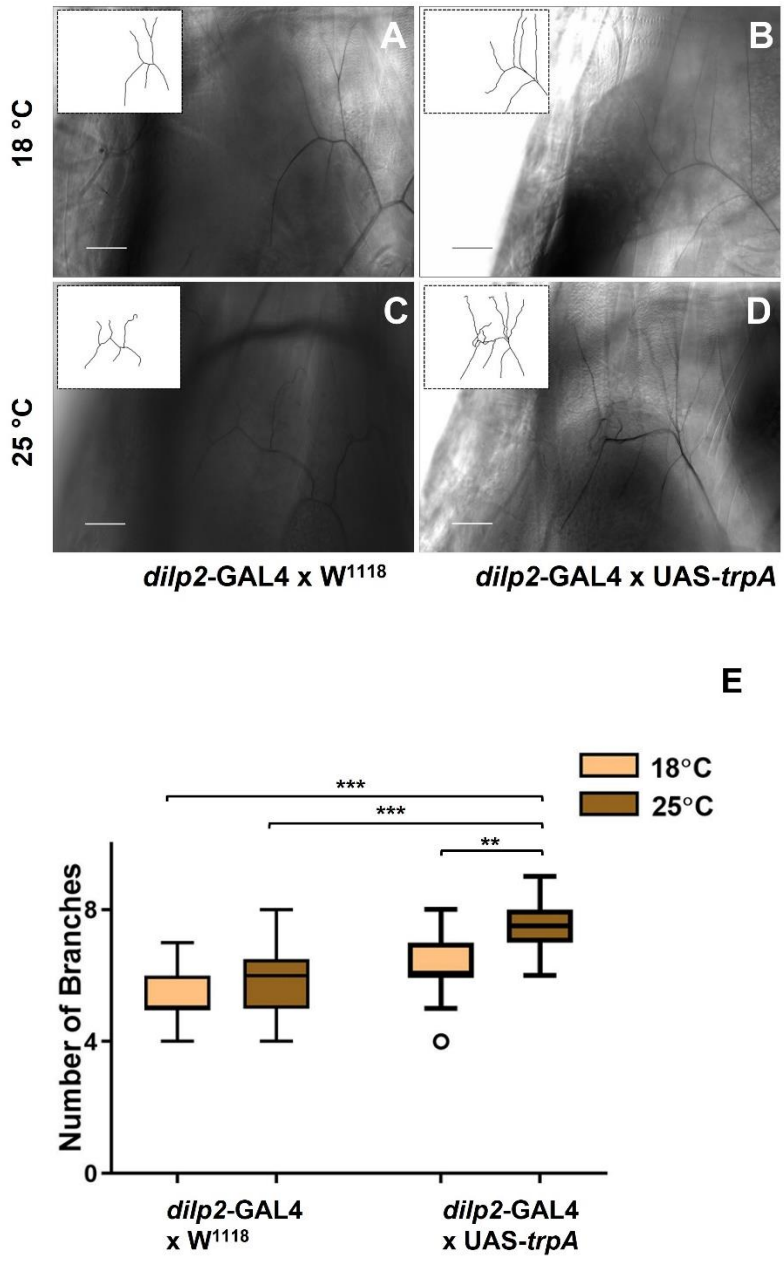



**Figure 25. Thermogenetic activation of Dilp2 neurons using TrpA promoted tracheal terminal cell plasticity.** A-B) Representative tracheal branching of (A) control larvae (dilp2-GAL4 x W1118), and larvae with thermogenetically activated dilp2 neurons (dilp2-GAL4 x UAS-TrpA1), maintained at 18 °C. (B) under normoxia. C-D) Representative tracheal branching of control (C) and dilp2-GAL4 x UAS-TrpA1 (D) under hypoxia. E) Quantification of branching in control and dilp2-GAL4 x UAS-TrpA1 under normoxia and hypoxia. The scale bar in all images is 50µm. n=15-16. Represented are means ± SD. $p<0.001$(ANOVA), Tukey's significance test, Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05,

Larvae maintained under 25°C, wherein the TrpA1 channels were activated in DILP2 neurons, showed increased terminal cell branching (7.6±0.88). The number of terminal branches in the control larvae (6.2±1.0) maintained at 18 °C remained within the norms (Fig. 25). These data suggest that heat impulse induced the secretion of dILP2, possibly leading to its release in the hemolymph and thus inducing terminal cell branching.

### 3.11 Use of TU-tagging yielded tracheal terminal cell-specific mRNA

To test the ability to label RNA in the tracheal terminal cells biosynthetically, *Drosophila* larvae were fed fly food infused with 4TU. After delivering 4TU, we monitored its incorporation into RNA by purifying total RNA, performing thio-biotin coupling, and using streptavidin-HRP to detect labeled RNA (see methods). To confirm that thio-labeled RNA was from UPRT-expressing cells and demonstrate the utility of TU-tagging for cell type-specific

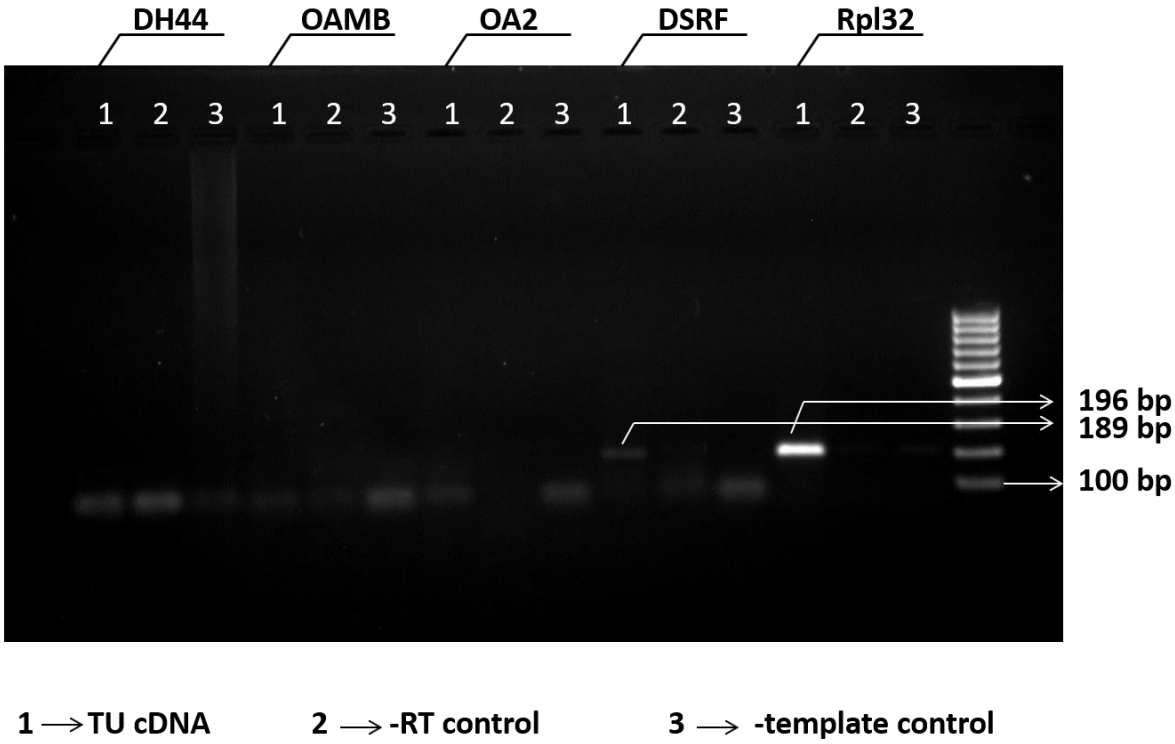



**Figure 26. TU-tagging: Agarose gel electrophoretic analysis of RT-PCR products for detecting TU cDNA.** Three brain specific genes DH44, OAMB, OA2 were used as negative markers (would detect non terminal cell cDNA, if present any). TTC specific gene DSRF was used as a positive marker to detect TU labelled cDNA. Rpl32 was used as an internal gene control. There were no cDNA bands identified corresponding to *dh44*, *oamb*, or *oa2*. DNA bands were visualized for *dsrf* matching its length(189bp) and rpl32(192bp)**.**

RNA isolation, we purified TU-tagged and untagged RNA and detected them using RT-PCR. Primers for tracheal terminal cell-specific gene *dsrf* were used to detect the presence of TU-tagged RNAs. Three different genes that were expressed in tissues other than the trachea were

used to confirm if there was a nonspecific expression of UPTR and the control reference gene. The genes chosen were DH44- central nervous system; embryonic abdominal segment; peptidergic neurons; and the ventral midline of the embryo, OAMB- G-protein coupled receptor - expressed in mushroom bodies, OA2- is expressed in several structures, including central nervous system; dorsal closure embryo; embryonic head sensory system; epithelial tube; and imaginal disc. In RT-PCR reactions, the housekeeping gene Rpl was used as internal control gene. cDNA band of matching 189 bp was obtained on the agarose gel, which corresponded to the specific length of the *dsrf* gene fragment, seemingly confirming that the TU tagging method enriched terminal tracheal cell-specific mRNA (Fig. 26). No DNA bands were visualized on the agarose gel for any other genes, *dh44*, *oamb*, or *oa2*, chosen as markers for the nonspecific tissues, suggesting that there was no UPTR expression in non-tracheal.

# 4 DISCUSSION

Organisms and their cells are exposed to various stress events arising from physiological processes and external stimuli. All organisms have evolved cellular stress response mechanisms, ensuring survival in challenging conditions [165]. Adaptation to stress is achieved mainly through the induction of highly conserved cellular response processes, which are mediated by stress-activated signaling pathways that define cell fate. However, while cellular stress response mechanisms support cellular functions and maintain microenvironmental and organismal homeostasis, they can eventually lead to impairment over time, leading to aging and diseases such as diabetes, heart disease, neurodegeneration, and cancer[166]. $O_2$ is an essential component required for the maintenance and growth of aerobic animals, similar to the essentiality of what is classically considered nutrients. In most animals, low oxygen availability is particularly deleterious, can result in rapid tissue damage, and may be lethal. Oxygen deprivation is a hallmark of many pathological conditions like stroke, ischemia, and cancer. Added to cardiovascular stress, hypoxia also alters the body's energy metabolism. Hypoxia also modifies adipose tissue circulation, which has a vital role in its metabolism and consequently affects obesity and diabetes. Nevertheless, some animals have evolved to live in oxygen-deprived conditions and consequently exhibit phenotypes that can withstand hypoxia. In humans, insufficient oxygen supply caused by reduced oxygen saturation of arterial blood results in cardiovascular system modifications to deliver more blood to tissues to compensate for reduced oxygen delivery [167]. Understanding the dynamics and reciprocal interplay between stress responses and hypoxia is critical for developing novel therapeutic strategies that exploit endogenous stress combat pathways against hypoxia-associated pathologies[168].

In their natural ecology, *Drosophila* larvae grow on decomposing food rich in microorganisms, which probably contribute to a low-oxygen local environment and have evolved metabolic and physiological mechanisms to tolerate hypoxia. In *Drosophila*, the adaptation to hypoxia is achieved through mechanisms that enhance oxygen delivery, such as the expansion of the spiracular openings, that drive delivery of Oxygen to the whole organism [169]. The expression of HIF1 induces an increase in the diameter of tracheal tubules and causes the expansion of cells that directly makes contact with the target tissues, the tracheoles [170]. The tracheal terminal cells respond to hypoxia by extending cytoplasmic tubular processes, the terminal branches, toward the hypoxic tissue [38]. Thus, terminal cell remodeling in *Drosophila* is controlled by the evolutionarily conserved HIF-1α/Sima pathway, similar to tip cell remodeling

of mammalian blood vessels. Here we report the importance of modulation of the FoxO signaling pathway as a regulator of tracheal terminal cell branching during sensitivity to hypoxia and nutrition. In particular, we find that the expression of FoxO must be at a specific physiological range for animals to promote the branching phenotype. We further show that FoxO-mediated remodelling of terminal branching can be induced by IIS signaling. Our findings implicate that the expression of FoxO in TTCs in larvae is critical in coordinating phenotypic plasticity and regulating the response to changing oxygen levels. Along with the well-studied adaptations to hypoxia that are mediated by HIF-dependent changes in gene expression, it seems likely that FoxO mechanisms might supplement the transcriptional response to hypoxia by regulating TTC plasticity.

### 4.1 Hypoxia and nutritional availability as two cues that regulate tracheal terminal cell plasticity in *Drosophila* larvae.

Organisms adapt their metabolism and growth to the availability of nutrients and oxygen, which are essential for development and survival. Yet, the mechanisms by which this adaptation occurs are not fully understood. Mammalian vasculature is analogous to the tracheal system in *Drosophila*, and both systems are known to respond dynamically to hypoxic conditions to maintain a constant supply of oxygen to peripheral tissues [171]. A study published by *Linneweber et al.* (2014) reveals that tracheal plasticity can also be regulated by nutrient availability and not only respond to nutrient cues but also leave direct long-lasting changes in tracheal morphology that provide metabolic benefits for the organism [100]. The observations from the current study indicate that the tracheal system of third-instar wild-type larvae responds to hypoxia (5% $O_2$), forming the pattern of terminal branch sprouting displaying terminal cell plasticity (Fig.14. B,C), as reported by earlier studies [38], [97], [172]. In this study, we show that a reduction in dietary yeast (3.5% yeast) led to reduced branching of the tracheal terminal cells in the body wall, and an increase in dietary yeast (10% yeast) led to an increase in the branching of tracheal terminal cells (Fig14. D,E ) and thus the results are in par with the study by *Linneweber et al.* (2014) [100]. The nutritional plasticity did not affect overall developmental timing or growth, suggesting that it occurred independently of tracheal development.

In *Drosophila,* tracheal development is assumed to depend on hard-wired developmental cues during embryonic stages. Later, in larval stages, tracheal terminal branching is driven by local hypoxia in the target tissues [38]. The effect of hypoxia on terminal cell branch number is

mediated directly through Bnl/Btl signaling and has been studied extensively. In brief, most tissues sense hypoxia through *sima*/*HIFα*, which is expressed but quickly degraded at normal oxygen levels [64], [84], [93], [173]. When sufficient oxygen is unavailable, Sima protein levels increase due to the inactivation of various factors that mediate its degradation. Sima then functions in cooperation with Tango to upregulate Bnl expression and secretion. Consequently, terminal cells respond by sprouting more branches [98]. This straightforward view is complicated by the fact that tracheal terminal cells respond themselves to hypoxia. On the one hand, the Bnl expressed and secreted additionally by other tissues is detected by tracheal cells. On the other hand, larval terminal cells detect hypoxia themselves and respond by upregulating the expression of *btl* [99].

Besides hypoxia, as discussed earlier, a more recently recognized environmental factor governing terminal branches branching behavior is nutrition. Initial studies by *Linneweber et al.* (2014) showed that the percentage of yeast in the nutritional medium is concurrent with the number of branches on dorsal terminal branches and those traversing the gut, barring the terminal branches reaching the brain [100]. Though the mechanism by which the tracheal branches respond to nutrition is not yet elucidated, it is known that, in response to specific nutritional cues, a small subset of neurons in the brain are activated to regulate tracheal branching in an organ-specific manner, and at least one of the two nutrition-responsive neuronal subsets identified also responds to hypoxia. The same study showed that these neurons exhibited increased activity in response to yeast and hypoxia as reflected by a fluorescent $Ca^{2+}$-reporter, implying that regulation of TC branching depended on both conditions. [100]. The notion of a shared neuronal substrate for both nutritional and hypoxic stimuli in invertebrates is remarkably similar to the mammalian carotid body: the cluster of chemoreceptors that monitors arterial oxygen concentration and nutrient levels to regulate breathing and cardiovascular tone[174], [175]. Tumors often grow in nutrient- and oxygen-poor environments and angiogenesis is a strategy used to obtain nutrients and oxygen. Consequently, angiogenesis is a critical target in cancer treatment [176]. Various recent studies have focused on the parallelisms in cellular behaviors in tracheogenesis in *Drosophila* and angiogenesis in mammals, supported by genetic and molecular conservation. As in human cancers, *Drosophila* malignant cells suffer from oxygen shortage, release pro-tracheogenic factors, co-opt nearby vessels, and get incorporated into the tracheal walls. In fact, tracheogenesis has been considered a novel cancer hallmark in *Drosophila*, further expanding the importance of the fly model for studying diseases [177]. Thus, it is relevant to understand

the molecular circuitry associated with tracheogenesis with the shifting nutrient and oxygen availability in the context of the tumor.

## 4.2 FoxO signaling as a common regulation pathway for nutrition and stress response in tracheal terminal cells.

The transcription factor FoxO is a core regulator of cellular homeostasis, stress response, and longevity since it can modulate various stress responses upon oxidative stress, hypoxia, heat shock, and DNA damage[178]. In the current study, we focused on FoxO response to both nutrition and hypoxia-induced plasticity of tracheal terminal cells. FoxO activity is regulated by post-translational modifications that drive its shuttling between different cellular compartments, thereby determining its inactivation (cytoplasm) or activation (nucleus). Depending on the stress stimulus and subcellular context, activated FoxO can induce specific sets of nuclear genes, including cell cycle inhibitors, pro-apoptotic genes, reactive oxygen species (ROS) scavengers, autophagy effectors, gluconeogenic enzymes, and others[126]. Under hypoxic stress, the FoxO was localized more in the cytoplasm than the nucleus of the tracheal terminal cells, which indicates the inactive form of FoxO, while the translocation happened in the opposite direction in the rest of the trachea (Fig. 16). We further studied how FoxO influences the branching phenotype of the tracheal terminal cells by comparing the phenotype in wild-type larvae, FoxO-deficient larvae, and larvae with forced FoxO expression. In wild-type animals, the TTCs sense hypoxia and respond by producing extra-ramifications, and this behavior shares many features of that of the "tip cell" of growing blood vessels during mammalian angiogenesis [95]. In this study, in animals where FoxO was deficient or FoxO was overexpressed, the TTCs failed to show extra ramification under hypoxic conditions, which clearly depicts that FoxO has a vital role in the regulation of branching phenotype under hypoxia (Fig.17). A similar result was obtained when FoxO deficient and FoxO overexpressed animals were subjected to variable nutritional availability. The nutritional plasticity shown towards high yeast concentration by wild-type animals was not observed in animals with altered expression of FoxO (Fig. 18). The sprouting and growth of the terminal branches are carefully adjusted according to the Oxygen and nutrition availability, just as in the case of sprouting angiogenesis in mammals.

As discussed earlier, angiogenesis in mammals is facilitated by the HIF-1-directed migration of mature endothelial cells toward a hypoxic environment. This is done via HIF-1 regulation of vascular endothelial growth factor (VEGF) transcription, a central angiogenesis regulator

that promotes endothelial cell migration toward a hypoxic area[1]. In *Drosophila*, the formation of new branches depends on the HIF-1α homolog Sima and the HIF-propyl hydroxylase Fga, which acts as an oxygen sensor [99]. The study by *Centanin et al.* indicates that the tracheal system of *sima* mutant third-instar larvae is indistinguishable from that of wild-type individuals, including the pattern of terminal branches [88]. Thus, the results imply that if terminal branching during normal development was mediated by tissue hypoxia, the mechanism that regulates such a local response could also be Sima-independent. This leaves the possibility of the involvement of additional factors other than Sima that could be involved in terminal branch response to tissue hypoxia. In this scenario, supported by branching data from controlled FoxO expression studies, it is plausible to speculate that FoxO could be another player acting independently of the HIF pathway in regulating terminal branching driven by tissue hypoxia. In mice, it has been shown that Endothelial-restricted deletion of FoxO1 induces a profound increase in EC proliferation that interferes with coordinated sprouting, thereby causing hyperplasia and vessel enlargement. Conversely, forced expression of FoxO1 restricts vascular expansion and leads to vessel thinning and hypobranching [179]. However, in mice, the FoxO regulation might be more complex due to the presence of more than one f FoxO gene; hence, the deletion of FoxO1 in mice might not implicate the complete absence of FoxO activity. Though the FoxO1 deletion in mice and FoxO deficiency in *Drosophila* did not have a similar outcome on the branching process, this study reinforces our finding that FoxO has a role in regulating terminal cell branching in *Drosophila* larvae.

### 4.3 The physiological range of FoxO expressed during hypoxia is essential for tracheal terminal cell plasticity.

We have demonstrated that during hypoxia, FoxO is localized in the cytoplasm of TTCs, whereas the reverse was observed in the rest of the tracheal cells. Despite FoxO deficiency and FoxO overexpression causing stunted growth of the terminal branch, there was an induction of branching when FoxO expression was partially reduced using RNAi in the terminal cells (Fig. 19), indicating that the physiological level of FoxO expressed or present is very significant for the induction of branching. The rapid induction of FoxO and its translocation to the nucleus observed in the dorsal trunk, primary and secondary branches of the trachea could be described as one way that FoxO provides protection in low oxygen through initiating an immune-like response. In *Drosophila*, the two critical pathways that respond to pathogen infection are the Toll and the immune deficiency (Imd) pathways. Activation of the Imd pathway leads to the nuclear translocation of the NF-κB transcription factor Relish to activate the expression of

effector proteins, including antimicrobial peptides (AMPs) [180]. Hypoxia can also mount an immune-like response and lead to the translocation of Relish. Additionally, there is evidence that hypoxia-induced Relish via FoxO and this response was required for hypoxia tolerance [181]. A recent study from our group that used *Drosophila* as a model to match the remodeling of airways associated with chronic lung inflammatory diseases revealed that triggering the Imd pathway in tracheal cells induced organ-wide structural remodeling. The study that structural remodeling of tracheal epithelium caused by hypoxia does not depend on the Imd signaling cascade's canonical pathway acting on NF-κB activation but relies on the activation of yet another segment of the Imd pathway that diverges downstream of Tak1 and constitutes activation of JNK and FoxO signaling [156]. These studies, along with others[182], [183], suggest that the immune-like response caused by the rapid induction of FoxO may be a protective mechanism in low oxygen in *Drosophila*.

In contrast to this immune response mechanism, the cytoplasmic localization of FoxO in the TTCs can be seen as a much slower adaptive response developed to cope better in an oxygen demanding situation. This could be perceived as a long-lasting situation built slowly in an oxygen demanding environment, where a physiologically relevant level of FoxO expression is achieved that might allow the sprouting of new branches in the terminal cells to accomplish better utilization of oxygen. It is possible that FoxO directly regulates some of the genes involved in terminal cell sprouting. Centanin and colleagues demonstrated that the hypoxia-induced sprouting of excess terminal branches is regulated by the accumulation of the Sima in terminal cells, leading to the induction of *breathless* [88]. There is evidence that FoxO was still relocalized to the nucleus in fat body cells in *sima* mutant larvae exposed to hypoxia [184], suggesting that the FoxO response mechanism is entirely independent of Sima. Hence it seems likely that FoxO activity in the terminal cells might also be independent of the classic HIF1α response and could act parallel to the HIF response. From the FoxO-RNAi data, it is apparent that the expression of FoxO in the terminal cells must be regulated tightly to achieve the optimal expression level to display the plasticity of the terminal cells.

## 4.4 FoxO at physiologically relevant level can alter some of the hypoxia-induced phenotypes

When larvae with FoxO-RNAi in the terminal cells were subjected to hypoxia, the growth restriction was significantly reduced from the control larvae in hypoxia (Fig.20). This could be because larvae with reduced FoxO expression also had high tracheal terminal cell coverage with improved oxygen delivery. Thus, the results suggested that reducing FoxO in the terminal

cells increased the terminal branching and could help the larvae cope better metabolically under hypoxic conditions. Though the results suggest that FoxO function in the larval tracheal system may reverse hypoxic growth restriction by improving oxygen delivery, we could also consider that the trachea systemically controls growth independent from its oxygen delivery functions. Few studies have demonstrated that the trachea can release factors to regulate specific biological processes unrelated to oxygen delivery function [185][186]. For instance, the larval trachea system produces and releases antimicrobial peptides (AMPs) into circulation as part of the organism's innate immune response upon bacterial infection [186]. Another study has indicated that the *Drosophila* tracheal system produces a Decapentaplegic (Dpp) concentration gradient that triggers Dpp signaling in enterocytes to regulate adult midgut homeostasis [185]. From these findings, we may also propose that hypoxia may inhibit tracheal growth factor production and release and could be regulated via FoxO signaling to control growth systemically.

In *Drosophila*, lipids are stored inside intracellular droplets in larval fat body cells. Under normal feeding conditions, nutrients accumulate in larval fat body cells and cause the tissue to become opaque. However, during starvation, the opacity of the tissue decreases as lipids are mobilized from the fat body [187][188] and accumulate in oenocytes [162]. Hypoxia also reduces the opacity of the fat tissue, like starvation but is not up took the oenocytes [102]. Our study also showed that wildtype larvae reared under hypoxic conditions resulted in decreased fat body opacity (Fig. 21B; compared to 21C), similar to that previously observed in a nutrient-deprived fat body [188]. Bodypi staining of larval fat bodies showed that hypoxia caused lipids in this tissue to aggregate in larger droplets (Fig. 22B), as compared to those observed in the wildtype control (Fig. 22C). However, hypoxia caused the loss of distinct cell boundaries in the larval fat body while maintaining homogeneity in lipid droplet size. Given that larger lipid vesicle aggregation in the fat body indicates efflux of lipids into the hemolymph [162], we investigated whether these lipids mobilized from the fat body showed a change in a pattern where FoxO was down-regulated in the terminal cells. When subjected to hypoxia, these larvae showed the fat body with large droplets of fat bodies, but the lipid droplets were less aggregated than wild-type larvae under hypoxia. Also, the cell boundaries of cells in the fat body in these larvae under hypoxia were more distinct than its wild-type control in hypoxia. This might indicate that the lipid in the fat body of larvae with dFoxO downregulated in the TTCs is not readily immobilized or effluxed into the hemolymph as that of the wild-type larvae under hypoxia. This combination of enhanced fat body opacity and lipid aggregation in the fat body

exhibited by hypoxic larvae demonstrates that hypoxia induces lipid metabolism defects and could be making an improvement by inputs from regulated FoxO signaling. Studies indicate that very long-chain fatty acids have a role in waterproofing the larval trachea, implicating the importance of proper lipid metabolism in maintaining tracheal integrity [189][190]. Taken together, these data demonstrate that FoxO is at a regulated level of lipid metabolism defects associated with hypoxia.

### 4.5 FoxO-dependent TTC plasticity is not regulated via JNK signaling

FoxOs respond to a wide range of external stimuli and are regulated through several posttranslational modifications (PTMs), including phosphorylation, acetylation, ubiquitination, and methylation. These PTMs occur in response to different cellular stresses, which in turn regulate the subcellular localization of FoxO family proteins, as well as their half-life, DNA binding, transcriptional activity, and ability to interact with other cellular proteins[126]. c-Jun N-terminal kinases (JNKs) are a family of protein kinases that play a central role in stress signaling pathways implicated in gene expression, neuronal plasticity, regeneration, cell death, and regulation of cellular senescence. Under different stress conditions, FoxO can be phosphorylated by JNK and imported into the nucleus to exert its function as a transcription factor. *Drosophila* homolog of mammalian c-Jun N-terminal kinase (dJNK) is called *basket* (*bsk*) [191], and the JNK pathway is relevant for the control of immune responses in the fly airways epithelial immune system [72].

As discussed earlier, Wagner *et al.* showed that triggering the Imd pathway in tracheal cells induced organ-wide structural remodeling of the fly airway system. The study revealed that structural remodeling involving disorganization of epithelial structures and epithelial thickening depended on the activation of the Imd pathway and comprised JNK-mediated activation of FoxO signaling [156]. However, in the current study, suppressing the JNK activity by expressing $bsk^{DN}$ did not influence the branching phenotype of the terminal cells either under normoxia or under hypoxia, implying that the mode of regulation of FoxO in the terminal cells is not regulated via the JNK pathway (Fig. 23). In this scenario, the irrelevance of JNK activity in the terminal cells is also in agreement with the fact that FoxO under hypoxia is more localized in the cytoplasm than in the nucleus of the terminal cells, as opposed to the rest of the cells in the trachea.

## 4.6 Activation of the dIlp Neurons Promotes tracheal terminal cell branching, suggesting FoxO response is regulated via the Insulin signaling pathway

Multiple signaling pathways such as the insulin/IGF-1 signaling, TOR signaling, AMPK pathway, JNK pathway, and germline signaling are integrated with FoxO signaling in regulating critical functions involved in cell metabolism, growth, differentiation, oxidative stress, senescence, autophagy, and aging [192]. Among these, it is clear that IIS pathways are exquisitely sensitive to changes in nutrient levels and play a pivotal role in regulating responses to physiological changes [193]. However, there is also abundant evidence that they are integrating and responding to a broad suite of environmental cues in addition to nutrient status. The systemic regulation of insulin signaling has been demonstrated to be an essential adaptive response to stress. In the context of this study, taking into consideration that (i) the IIS pathway is the central nutrient-sensing pathway, (ii) FoxO is a critical downstream point of regulation in the IIS pathway, and (iii) terminal cell branching is both Oxygen and nutrient-sensitive, we hypothesized that the tracheal cell plasticity in *Drosophila* larvae is regulated via the canonical IIS/FoxO pathway and could respond to varying nutrient and oxygen availability.

IIS in *Drosophila* is facilitated by the insulin-like peptides secreted by the insulin-producing cells (IPCs) in the larval brain [164][194]. The Dilps are released into the hemolymph and bind a single insulin receptor (InR) to activate the insulin and insulin-like growth factor signaling (IIS) cascade [195]. Reducing systemic IIS either by ablating the IPCs or removing key effectors results in a decreased growth rate, developmental delay and metabolic dysfunction, characterized by a ‘diabetic-like’ phenotype in *Drosophila* [164], [196]–[198]. We used the trypA channel to elicit the Dilp2 neurons and induce insulin production from the IPCs of the larval brain. Induction of insulin production from the larval brain significantly increased terminal tracheal sprouting, mimicking the phenotype observed in larvae subjected to oxygen deprivation and nutrition abundance (Fig. 3E; compared to 3D). The increase in insulin signaling mediated by the expression of trpA in Dilp2 neurons may promote the organism's adaptive response to hypoxia, as demonstrated by the increased sprouting of branches. But, some studies indicate that hypoxia increased the retention of Dilp2 in the insulin-producing cells of the larval brain, associated with a reduction of insulin signaling in peripheral tissues [102]. In larvae, mutant for the InR ligands *ilp2, 3* and *5*, it was demonstrated that dorsal TCs had severely reduced branch numbers. At the same time, the TCs in most parts of the gut were unaffected; moreover, overexpressing InR increased dorsal TC branching by around 20% [102]. A neuronal population was identified for those TCs that tracheate the hindgut, which

directly regulates TC branching in this region by secreting Ilp7 [100]. From these studies it may be assumed that the FoxO regulation could be taking place at insulin receptor level by simply regulating the expression of InR (Fig. 27). The finer details of insulin signal reception by these cells thus require further experiments to dissect the point of regulation because this could be a case where careful titration of insulin levels is essential for an organism to adapt to dynamic environmental inputs.

A recent study indicates that a fat body-derived hypoxia signal represses insulin release from the brain. It has been demonstrated that in *Drosophila,* the availability of nutrients is remotely sensed in fat body cells and conveyed to the brain IPCs by a humoral signal controlling ILP release [199]. Furthermore, hypoxia activates a fat-tissue oxygen sensor that remotely controls the secretion of insulin from the brain by inter-organ communication [200]. These Studies show that the fat tissue could act as the primary sensor of nutrient and oxygen levels, directing an adaptation of organismal metabolism and growth to environmental conditions. It is also known that conditioned medium from hypoxic adipocytes alters insulin signaling in muscle [78], suggesting an inflammatory signal that may regulate systemic insulin inputs. In *Drosophila*, the closest homologs to cytokines are the family of Unpaired (Upd) ligands, which signal through JAK-STAT receptors. HIF-1α has been known to regulate STAT signaling to modulate insulin sensitivity [201]. This, combined with the finding that Upd2 regulates Dilp secretion in *Drosophila*[202], further supports the conservation of coordinated insulin signaling in flies and humans as an adaptive response to environmental stressors, such as hypoxia. It has been demonstrated that insulin can activate HIF-dependent transcription, both in *Drosophila* S2 cells and in living *Drosophila* embryos, and the effect of insulin on HIF-dependent transcriptional induction is mediated by PI3K-AKT and TOR pathways. The study also demonstrated that overexpression of dAKT and dPDK1 in normoxic embryos triggered a significant increase in Sima nuclear localization, mirroring the effect of a hypoxic treatment [203]. The integration of these pathways must be considered to understand the phenotypic plasticity regulated by different cues properly.

Though Insulin's primary role is regulating systemic carbohydrate and lipid metabolism [203], it also has a vital role in vascular function, for example, promoting vasodilation and tissue perfusion [204] in mammals. The loss of endothelial insulin receptors, or disruption of their signaling function, induces endothelial dysfunction, hypertension, and atherosclerosis [205]–[207]. Sprouting angiogenesis, the formation of new capillaries, is a fundamental element of vascular biology intrinsically linked to metabolism [208]. In this highly conserved process,

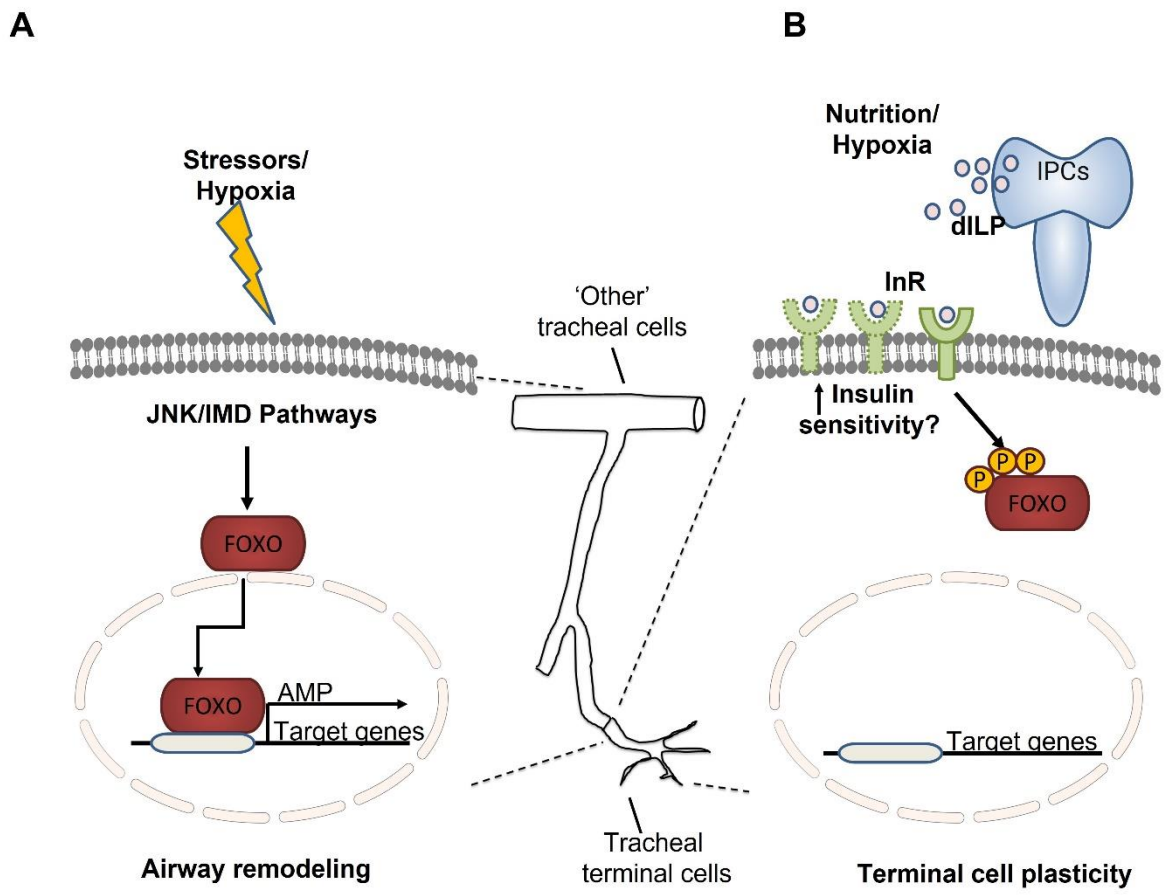



**Figure 27. Proposed model of FoxO regulation in the *Drosophila* tracheal cells**. Airway remodeling that occurs from chronic activation of the immune system/stress are mediated by the segment of the IMD pathway, involving the JNK pathway and subsequent activation of the transcription factor FoxO(A). A different mode of activity of FoxO in the TTCs which is different from the mode of action in the rest of the trachea(B). Under low Oxygen availability/nutrition abundance FoxO is retained in the cytoplasm, leading to inhibition of FoxO transcriptional activity, but allowing tracheal terminal cell plasticity. Induction of Dilp secretion from the larval brain also induces plasticity of the terminal cells. Though it is possible that increased insulin activity can negatively regulate FoxO retaining it in the cytoplasm, other studies reveal that reduced environmental oxygen levels act on the insulin-producing cells (IPCs) to down-regulate Dilp transcription and to block DILP release. One plausible interpretation is that regulation of insulin signaling is possible at the receptor level (InR) making the TTCs more sensitive to reduced insulin signaling or the regulations might include targets downstream of InR.

endothelial tip cells emerge from existing vessels, followed by proliferating stalk cells that extend the sprout and form a lumenized vessel; these neovessels form a network that remodels to meet local demands for oxygen and metabolite transport [209]. Insulin promotes angiogenesis *in vitro* and *in vivo* in mammals [210]–[214]. In *Drosophila*, the regulation of tracheal branching morphogenesis is significantly analogous to that of mammalian angiogenesis, with *Drosophila* FGF/FGFR, encoded by the branchless (*bnl*) and breathless (*btl*) genes, respectively, carrying out the functions of VEGF/VEGFR in

angiogenesis[215], [216]. Understanding the changes brought about by hypoxia in this model system may allow a greater understanding of the influence of insulin on angiogenesis and human disease associated with tissue hypoxia, such as obesity and diabetes.

### 4.7 TU Tagging as a method to isolate terminal cell-specific RNA must be re-examined and calls for the use of alternative methods

Gene profiling techniques make possible the analysis of transcripts from organs, tissues, and cells with unprecedented coverage and is an essential determinant of cell fate and function. While techniques that quantify mRNAs (RNA-seq, microarrays) allow investigation of gene expression, the quality and type of information obtained may be limited by the method of RNA purification [217]. Methods for isolating cell type-specific mRNA generally fall into two categories: physical isolation or tagging and capturing RNAs. Techniques based on physical isolation (fluorescence-activated cell sorting, laser-capture microdissection, INTACT (nuclei tagged in a specific cell type)) disrupt the cell's environment and may affect mRNA transcription or decay. Most tissue-specific transcriptome analysis methods available for *Drosophila* are based on dissection techniques, are time-consuming, and lead to variability due to dissecting irregularities and small sample sizes. Also, these methods have limitations, including unwanted detection of transcripts from other tissue types and underrepresentation of rare transcripts. Besides, subjecting some tissues to dissection is challenging, and cell-specific analyses cannot be carried out precisely [218]. Various approaches that eliminate dissection have been developed to create profiles of specific cells in complex tissues, including methods based on isolating cells of interest after tissue dissociation. Such strategies include Fluorescence-activated cell sorting **(**FACS) [219], manual sorting of fluorescent cells, and immunopanning of dissociated cells that involves FACS and not [220][221]. Although each of these methods has exclusive advantages, they all lack the ability to profile cells *in situ* without introducing experimental noise resulting from tissue fixation, dissociation, and incubation. The challenges mentioned above call for powerful alternative methods to profile the transcriptome of any cell population in *D. melanogaster*.

Using the 4TU/UPRT-based biosynthetic labeling method called "TU-tagging," it is possible to restrict UPRT expression in a multicellular organism spatially; 4TU will be modified and subsequently incorporated into nascent RNA only in cells expressing UPRT [139]. This way, even in the RNA isolated from the whole organism, RNA from the cells expressing UPRT can be retrieved by purifying TU-labeled RNA. This method would thus be beneficial for isolating

RNA from cell types that are difficult to isolate by dissection or dissociation methods, such as subsets of neurons or glia in the CNS. Due to these reasons, the use TU-tagging method for the isolation of mRNAs from tracheal terminal cells seemed ideal because they are relatively scarce in number (~300 cells) and structurally challenging to dissect and separate from the adhering tissue. In this study, we used Dsrf-Gal4 to drive the expression of UPTR in the terminal cells, which came together in the F1 progeny to incorporate 4TU into the transcribing mRNA. We used terminal cell-specific primers as positive controls in cDNA PCR to confirm that the mRNA thus isolated is from the tracheal terminal cells. Agarose gel detected positive bands for terminal cell marker *dsrf*, thus confirming the presence of TTCs-specific TU incorporated transcript. As negative controls, we used some primers specific for the brain (non-specific tissue), and the cDNA PCR did not yield any bands specific for any of the brain-specific primers used. Though there were no positive bands for any markers used as a negative control, it is not possible to eliminate the chance of the presence of mRNA from non-specific tissue, merely from cDNA PCR. An essential feature of the TU-tagging method is that only newly transcribed RNAs are labeled. Thus, the percentage of labeled cellular RNA will depend on the duration for which labeling is carried out, implying that for a shorter labeling duration, there could be a considerable amount of RNA that is not TU-labelled. Even though long labeling periods should work well for isolating the majority of RNA present in a particular cell type, short labeling periods could be useful in detecting variations in gene expression at consecutive time points in specific cell types because newly synthesized RNA could be separated from the whole cellular RNA. This would be particularly beneficial in studying rapid changes in gene expression following a particular developmental or physiological event [139].

However, in subsequently published studies, it was found that TU feeding can lead to background incorporation into mRNA and is toxic to flies. Due to this reason, it was decided not to proceed with further processing of TU-tagged mRNA for transcriptome analysis. TU-tagging has been adapted in various model organisms and has proven useful in several systems [139]–[141]; however, given endogenous and alternative pathways for uracil incorporation, the specificity for labeling in this technique remains ambiguous [142]. An alternative tagging method called ‘EC-tagging’ that could circumvent the downside caused by endogenous pathways of TU incorporation was developed recently. This method requires the activity of two enzymes: cytosine deaminase (CD) and UPRT [218]. The sequential action of these enzymes converts 5-ethynylcytosine (EC) to 5-ethynyluridine monophosphate, which is subsequently incorporated into newly synthesized RNAs. The ethynyl group allows efficient

detection and purification of EC-tagged RNAs. It has been demonstrated that *Drosophila* can be engineered to express CD and UPRT. It can be used with high sensitivity and specificity to obtain cell type-specific gene expression data from intact *Drosophila* larvae, including transcriptome measurements from a small population of central brain neurons [218]. EC-tagging could provide advantages over TU tagging and could help investigate the role of differential RNA expression in Tracheal terminal cell identity under hypoxia. However, all tagging methods that involve incorporating tags through feeding should be carefully evaluated and designed for experiments that include hypoxia because hypoxia prompts the larvae to crawl away from the food. Feeding is significantly reduced, whereas control animals will continue to feed under normal conditions.

Another alternative RNA trap method that could be considered for use in the future is the translating ribosome affinity purification (TRAP) method[221] that selectively expresses the enhanced green fluorescent protein (GFP)-tagged ribosomal protein (RP), GFP: RpL10A in targeted cell type, using the versatile binary GAL4/UAS system [222]. EGFP-tagged ribosomes can be affinity-purified with EGFP antibodies. With TRAP, cell type-specific translating mRNA can be rapidly and efficiently isolated, allowing gene expression profiling of the specific cell population. TRAP circumvents the long time required for fluorescence-activated cell sorting that could change the gene expression profile. Furthermore, since only the specific cells express the GFP: RPL10A fusion protein, the isolated mRNA is devoid of contamination from the surrounding cells and other cell types. The affinity-purified mRNA method is a superior choice because of its high quality and allows downstream PCR- or high-throughput sequencing-based gene expression analysis.

## 4.8 Summary and Future perspective

Plasticity is a fundamental biological process that ensures that individuals' morphology, behavior, and physiology match their environment. Understanding the mechanisms that regulate the extent of trait plasticity is vital because understanding how phenotypic plasticity is regulated has essential consequences for studying diseases that result from changes in plasticity. Tracheal terminal cells of the fly airways system are plastic in nature, possess the ability to read the oxygen availability of the environment, and respond appropriately to compensate for the oxygen starvation in the surrounding tissue. The master regulator of the transcriptional response to hypoxia is HIF-1, mediating cellular and physiological adaptations to hypoxia, including genes involved in hypoxia-driven tracheal branching. This study

identified FoxO as an additional regulator of branching phenotype both in response to hypoxia and excess nutrition, the two known external cues that promote terminal cell branching. Deregulating the FoxO expression hampered the ability of the TTC to exhibit plasticity. In the future, it would also be interesting to evaluate if FoxO acts parallel to the HIF pathway and if there is a crosstalk between both pathways. FoxO localization studies revealed that the mode of action of FoxO in the terminal cells differs from its mode of action in the rest of the trachea. However, the FoxO activity in the terminal cells depended on JNK activity. On the other hand, inducing the IPCs to activate IIS signaling promoted the branching phenotype. Considering that other studies have shown retention of DILP in the brain during hypoxia, further studies are necessary to establish the mode of action of the IIS pathway and how DILPs feeds the input to regulate FoxO signaling in the TTCs. Future work should be conducted to establish if these DILP-secreting *Drosophila* neurons can sense oxygen and nutrients directly and whether they do so by using mechanisms akin to those described in the carotid body. This would further support the existence of an evolutionarily conserved link between oxygen and nutrient neuronal sensing. Another challenge to address is creating a transcriptome profile specific for the terminal cells and choosing methods that work best for the experimental setup. Though TU tagging yielded cell-specific mRNA, it doesn't manifest as an appropriate method because of the drawbacks posed by the method. An alternative RNA trap method that could be considered for use in the future is the translating ribosome affinity purification (TRAP) method that selectively expresses the enhanced GFP-tagged ribosomal protein (RP) in targeted cell type. A better understanding of processes involved in tracheogenesis and response to tissue hypoxia will also address mechanisms underlying angiogenesis and tumor hypoxia.

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## Declaration

I, Reshmi Raveendran, hereby declare that my doctoral thesis entitled "The role of transcription factor dFoxO in regulating tracheal terminal cell plasticity of the fruit fly *Drosophila melanogaster*" is my original work. This dissertation has not been submitted for the award of doctoral degree to another examining body. This thesis was prepared following the guidelines of Good Scientific Practice of the German Research Foundation. I also confirm that an academic degree has never been withdrawn.

Kiel, ----------

Reshmi Raveendran

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