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Gut Derived Cytokine Signaling Mediates Hypoxia Tolerance in Drosophila

Ding, Kate

UNIVERSITY OF CALGARY

Gut Derived Cytokine Signaling Mediates Hypoxia Tolerance in *Drosophila*

by

Kate Ding

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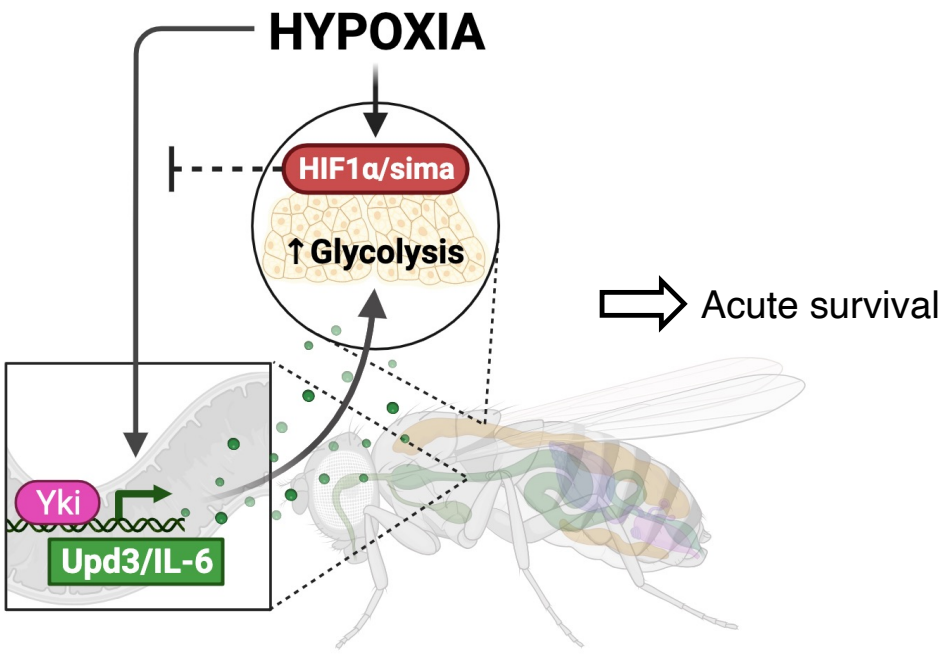
Abstract

Our cells and organs need oxygen to function. However, in some disorders such as stroke, heart disease and cancer, our tissues are deprived of oxygen. This lack of oxygen, known as hypoxia, leads to the tissue damage and deregulation that characterizes these diseases. Understanding how tissues respond to low oxygen is therefore an important question in health research. While extensive studies have identified hypoxic responses in cell culture, they leave open the question of how tissues and organisms deal with hypoxia. This is important since tissue-to-tissue crosstalk often underlies hypoxic responses in animals.

In their natural ecology, *Drosophila* have evolved to grow on rotting, fermenting food rich in microorganisms – an environment characterized by low ambient oxygen. Hence, they provide an excellent genetic model system to study how hypoxia influences physiology and development. Here I describe a mechanism for hypoxia tolerance in female *Drosophila* involving the cytokine Unpaired 3 (Upd3), a JAK/STAT pathway ligand and fly interleukin-6 homolog. I found that Upd3 whole-animal null mutant females, but not males, had reduced survival in hypoxia (1% O_2), indicating that the requirement for Upd3 signaling in hypoxia tolerance is sexually dimorphic. Using tissue-specific RNAi-mediated knockdown, I show that these survival effects require Upd3 production in the enterocyte cells of the fly intestine. I also identified transcription factors, Sima/HIF-1α and Yorkie/YAP, as regulators of Upd3 induction. I showed that intestinal Yorkie signaling was required for part of the Upd3 induction in hypoxia and that fat body Sima/HIF-1α, the classic hypoxia-induced factor required for low oxygen survival, acts non-autonomously to restrain excess intestinal Upd3 levels. I demonstrated that part of the lethality

seen in whole-body Sima mutants could be rescued by lowering Upd3 levels. Furthermore, I identified that the gut derived Upd3 targets the fat body to modulate glycolysis which is a necessary adaptation in hypoxia to promote tolerance (Graphical Abstract). These findings suggest Upd3 signaling must be tightly regulated in hypoxia: induction of Upd3 is required to mediate survival in low oxygen, but excessive Upd3 production can lead to a 'cytokine-storm'-like response which can cause lethality. Previous studies have demonstrated a link between HIF-1α and immune system modulation, thereby already implicating a role of hypoxia in immunity. However, with my discovery that gut derived Upd3 is upregulated in hypoxia and is restrained by fat body Sima/HIF-1α, I have uncovered a role for tissue-to-tissue communication in mediating hypoxia tolerance.



Graphical Abstract. Our model suggests that hypoxia induces Upd3/IL-6 from enterocytes in the *Drosophila* intestine via Yorkie/YAP signaling. Upd3/IL-6 then signals to the fat body to promote glycolysis which is required for overall hypoxia survival. The classical hypoxia-inducible factor, Sima/HIF-1α, functions in the fat body to restrain excess gut cytokine signaling. *Created with BioRender.*

Preface

This thesis is original, unpublished, independent work by the author, K. Ding.

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To all members of the Grewal lab, past and present, thank you for always providing a welcoming place to come to everyday. I will always cherish the memories of our lab lunches, coffee runs, and all other shenanigans.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
Bmm	Brummer
Bnl	Branchless
Bsk	Basket
Btl	Breathless
b-tub	β Tubulin
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CT	Threshold cycle
da	Daughterless
Def	Defensin
DN	Dominant negative
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
Dome	Domeless
Dpp	Decapentaplegic
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EB	Enteroblast
EC	Enterocyte
EEC	Enteroendocrine cell
eIF-2α	Eukaryotic transcription factor 2α
Fga	Fatiga
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOXO	Forkhead Box O
GFP	Green fluorescent protein

GlyP	Glycogen phosphorylase
GlyS	Glycogen synthase
GS	GeneSwitch
Hex-A	Hexokinase A
HIF	Hypoxia-inducible factor
Hop	Hopscotch
HRE	Hypoxia response elements
IL	Interleukin
IMD	Immune deficiency
IR	Inverted repeat
ISC	Intestinal stem cell
JAK/STAT	Janus kinase/Signal transducer and activator of transcription
JNK	Jun-N terminal kinase
LB	Luria-Bertani
LDH	Lactate dehydrogenase
Mad	Mothers against dpp
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
ODD	Oxygen-dependent degradation
OE	Overexpression
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
Pfk	Phosphofructokinse
PHD	Pyruvate dehydrogenase
PKG	Protein kinase G
Pyk	Pyruvate kinase
qRT-PCR	Quantitative real-time polymerase chain reaction
rcf	Relative centrifugal force
RNA	Ribonucleic acid

RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
rpm	Revolutions per minute
RpS24	Ribosomal protein S24
rRNA	Ribosomal ribonucleic acid
Sd	Scalloped
SEM	Standard error of the mean
SOCS	Suppressor of cytokine signaling
Sod1	Superoxide dismutase 1
TAG	Triacyl glyceride
TCA	Tricarboxylic acid cycle
TGF-β	Transforming growth factor beta
tkv	Thickvein
TOR	Target of rapamycin
Tot	Turandot
UAS	Upstream activating sequence
Upd	Unpaired
VHL	von-Hippel-Lindau
Yki	Yorkie

Nomenclature

Category	Example
Proteins	Upd3
Genes	*upd3*
Alleles	*upd3*$^{\Delta}$
Homologous chromosomes	Indicated by forward slash (/)
Heterologous chromosomes	Indicated by semicolon (;)
Wild-type chromosomes	Indicated by plus sign (+)

CHAPTER ONE: INTRODUCTION

1.1 Organismal Responses to Hypoxia

Animals often exist in environments where conditions such as temperature, nutrient availability, and oxygen levels fluctuate. Therefore, animals must develop mechanisms to tolerate changes in their environment to maintain homeostasis. While most animals, particularly humans and other mammals, cannot tolerate more than a few minutes of oxygen deprivation, some animals have evolved to tolerate this extreme stress (Haddad et al., 1997). For example, the naked mole-rat can tolerate up to 18 minutes of complete oxygen deprivation (anoxia) without evidence of injury (Park et al., 2017). It manages this through metabolic rewiring which involves switching to fructose-fueled anaerobic metabolism, circumventing negative feedback of glycolysis via phosphofructokinase to support viability (Park et al., 2017). Another extremely anoxia tolerant organism, the brine shrimp embryo, has been shown to tolerate up to 4 years of anoxia by apparent complete shutdown of metabolic programs (Clegg, 1997). The likely explanation for these divergent differences in tolerance to hypoxia in these animals is their ability to adapt their physiology.

1.1.1 *Physioxia*

Hypoxia is also a feature of normal physiology. The air we breathe contains ~20% oxygen, but our tissues and organs experience much lower levels than this (McKeown, 2014). For example, early fetal development occurs in very low oxygen levels in the womb. In adults, the physiological range of oxygen levels, termed physioxia, ranges from 2-6% depending on the tissue (McKeown, 2014). Despite this, most cell culture experiments are conducted at ambient

air oxygen levels (~20% O_2). At this level, cells are receiving much more oxygen than they would *in vivo* (Carreau et al., 2011). Most work in the field has identified hypoxic responses in cell culture, however they leave open the question of how tissues and organisms deal with hypoxia. This is an important consideration since non-autonomous, tissue-to-tissue crosstalk often underlies hypoxic responses in animals. Therefore, a clearer understanding of hypoxic responses at the tissue and whole-body level warrants studies in genetically amenable model organisms.

1.1.2 *Pathological Hypoxia*

Our cells and organs need oxygen from the air we breathe to function normally. In conditions of low oxygen, also known as hypoxia, animals experience tissue damage and deregulation of metabolic homeostasis. These are characteristic of diseases such as cancer, ischemia, and chronic lung diseases (Manoochehri Khoshinani et al., 2016; Sherwood et al., 1971; Semenza, 2011; Biddlestone et al., 2015). These hypoxic states can occur locally or on a whole-organism level. In solid tumors, cancerous cells have been shown to adapt to their local hypoxic conditions and become resistant to radiation and chemotherapy treatment (Manoochehri Khoshinani et al., 2016). Hypoxia-induced angiogenesis has also long thought to be important for tumor growth and metastasis (Sherwood et al., 1971). In ischemic cardiovascular disease, impaired adaptation to hypoxia is related to worse perfusion recovery and more severe tissue damage (Semenza, 2011). If lung function is impaired, whole-body hypoxia can occur. For example, in chronic lung disease like pulmonary hypertension, hypoxia is known to induce expression of genes in lung smooth muscle cells which increase contraction and proliferation of these cells. This further restricts blood flow and contributes to chronic hypoxia, worsening the disease (Semenza, 2011).

1.1.2.1 HIF-1α is a Master Regulator of Hypoxia Responses

HIF-1α as a transcription factor acts as a master regulator of oxygen homeostasis. It is highly conserved – most all plants and animals have a HIF-1α homolog. HIF is a heterodimeric complex of two basic helix-loop-helix proteins of the Per-ARNT-Sim (PAS) domain family consisting of HIF-1α and HIF-1β. HIF-1β is constitutively expressed, whereas HIF-1α is controlled at the level of protein degradation and its stabilization can be affected by oxygen availability. In normoxia, HIF-1α protein is made but rapidly degraded through oxygen-dependent prolyl hydroxylation and subsequent targeting for proteolytic degradation. However, in hypoxia, prolyl hydroxylase activity is suppressed, HIF-1α is stabilized and dimerizes with HIF-1β to translocate to the nucleus and bind hypoxia-response elements (HREs) to promote gene expression (Kaelin et al., 2008). Transcriptome analyses have shown that HIF-1 directly or indirectly controls expression of thousands of genes, including target genes of glucose metabolism, erythropoiesis, angiogenesis, cell proliferation and survival (Ali et al., 2015). Despite being expressed in almost all cell types in response to hypoxia, expression of target genes is cell-type-specific. The kind of response elicited by HIF-1α is therefore dependent on developmental and physiological programming of the cell. Based on transcriptome analysis, it is estimated that 1-5% of human genes which are induced in response to hypoxia are HIF-1-dependent (Semenza, 2003a).

A classic HIF-1 role is in the control of erythropoiesis and angiogenesis. The circulatory system delivers oxygen and nutrients to all cells. Upon oxygen deprivation, HIF-1 acts as a main transcriptional regulator of genes involved in erythropoiesis and angiogenesis. HIF-1 was initially discovered as a transcription factor for erythropoietin (EPO) in conditions of low oxygen

in the blood (Semenza & Wang, 1992). HIF-1 also participates in every step of angiogenesis. In the first step, HIF-1 directly targets vascular endothelial growth factors (VEGF) and their receptors. In addition to VEGFs, other factors such as placental growth factor (PGF), platelet-derived growth factor (PDGF), angiopoietins 1 and 2 (ANGPT1 and ANGPT2), and metalloproteinases (MMPs) participate in angiogenesis. These gene products play critical roles in the vascular response to hypoxia and ischemia (Semenza, 2014).

HIF-1 also plays critical roles in regulating cellular metabolism in low oxygen. One of the most well-studied metabolic adaptations to hypoxia is the upregulation of glycolytic metabolism. Glycolysis is the process by which glucose is converted into pyruvate. In normoxic conditions, pyruvate can be transported into the mitochondria to be metabolized through the TCA cycle which then fuels oxidative phosphorylation. But in low oxygen, when oxidative phosphorylation is disrupted, glycolysis is upregulated to provide a source of ATP and the pyruvate produced is converted to lactate. An extensive body of work has shown that HIF-1 is the regulator of this glycolytic switch by controlling expression of genes involved in glucose and glycolytic metabolism. In flies, one study showed that adults mobilize their glycogen stores to fuel glycolysis in a HIF-1α/Sima dependent manner (Y. Li et al., 2013). In conditions of low oxygen, HIF-1 directs a crucial switch from oxidative phosphorylation to anaerobic glycolysis. It transcribes glucose transporters GLUT-1 and GLUT-3 which are responsible for trafficking glucose into the cell. Through the process of glycolysis, glucose is then metabolized to pyruvate. HIF-1 also controls expression of glycolytic enzymes hexokinase, aldolase, phosphoglycerate kinase, enolase, and pyruvate kinase. Another target of HIF-1 is lactate dehydrogenase, which converts pyruvate to lactate (Semenza, 2010). Pyruvate dehydrogenase kinase, another HIF-1

target, inhibits pyruvate dehydrogenase to divert metabolic flux away from the TCA cycle and electron transport chain, as it converts pyruvate to acetyl-CoA to allow it to enter the TCA cycle. This allows cells to continue ATP generation without generating harmful ROS because of utilizing the electron transport chain in hypoxia (Kim et al., 2006). These metabolic effects of HIF-1 are particularly important in cancer cells which need to grow in a low oxygen microenvironment.

1.1.2.2 Role of HIF-1α in Disease

As previously mentioned, low oxygen levels are associated with various human diseases. Hypoxia regulation of HIF-1 is also implicated in a number of these diseases. One such example of this is activation of HIF-dependent angiogenesis in the tumor cell mass. As tumors grow and proliferate, oxygen supply within the tumor often becomes limited, resulting in intratumoral hypoxia (Zimna & Kurpisz, 2015). HIF-1 is subsequently activated and induces proangiogenic factors which allows new blood vessels to form and supply nutrients and oxygen to the tumor (Semenza, 2003b). In addition to angiogenesis, HIF-1 activates transcription of genes which play important roles in genome instability, immune evasion, metabolic reprogramming, metastasis, radiation resistance, and stem cell maintenance (Semenza, 2011; Zimna & Kurpisz 2015). Furthermore, overexpression of HIF-1α in primary tumors is shown to be associated with adverse outcomes in several common cancer types (Zhong et al., 1999). Ischemic heart disease (IHD) is another common pathology in which reduced tissue perfusion results in tissue hypoxia. In healthy individuals, this tissue hypoxia leads to activation of HIF-1 and proangiogenic factors, allowing normal blood supply to be restored (Semenza, 2014). Therefore, HIF-1 has become a potential therapeutic target for the treatment of these diseases.

1.2 *Drosophila* as a Model to Study Hypoxia Tolerance

Drosophila melanogaster, the common fruit fly, has evolved to tolerate conditions of low oxygen (hypoxia) in its different life stages. For instance, larvae burrow in fermenting fruit to feed and adults lay eggs on the same rotting fruit, characterized by high CO_2 and low O_2 levels. Flies are therefore quite tolerant to hypoxia and are useful for studies investigating mechanisms which underlie this tolerance. As previously mentioned, cell culture experiments have provided useful information on signaling pathways that are induced in hypoxia but are limited in their capacity to understand tissue-specific roles in hypoxia sensing and adaptation. Functional studies on tissue-to-tissue communication are also difficult to do in mammalian systems due to their complex genetics and physiology. On the other hand, flies have been well established as a model to study questions of systemic physiology because of the speed and ease of genetic manipulation. Moreover, many signaling pathways are conserved in a more simplistic form in flies, therefore allowing us to ask pertinent biological questions that are relevant to higher organisms (Bier, 2005).

Previous work from our lab used *Drosophila* as a model to investigate how flies can tolerate hypoxia. One of the ways flies do this is through co-opting the innate immune system. It was discovered that the IMD/Relish pathway, which normally responds to gram-negative bacterial infection, is induced and required for survival in hypoxia. When tested, our lab found the Toll pathway was not involved in the hypoxia response (Barretto et al., 2020). The third branch of innate immunity, the cytokine/JAK/STAT pathway, has not yet been studied in the context of

hypoxia. In this thesis, I use *Drosophila* to explore the role for the innate immune signaling pathway cytokine/JAK/STAT in tissue-to-tissue communication to promote hypoxia tolerance.

1.2.1 Drosophila Life Cycle and Physiology in Hypoxia

1.2.1.1 Lifecycle

Drosophilae belong to a class of holometabolous insects in which larvae undergo whole-body metamorphosis. Its lifecycle can be divided into four stages: embryo, larva, pupa, and adult. Embryogenesis lasts 24 hours, at which point the larvae hatch and immediately begin feeding. Over the course of four days, larvae in nutrient-rich conditions can increase their mass by 200-fold before entering the pupal stage and metamorphosis also lasting four days (Church & Robertson, 1966). At this point, a sexually mature adult fly is formed. Their lifecycle is short, making it possible to generate large numbers for genetic, biochemical, and molecular experiments.

1.2.1.2 Respiratory System

In *Drosophila*, oxygen is delivered directly from the air to tissues through a branched network of trachea known as the tracheal system. Pairs of spiracles located on the thorax and abdomen open and close through valves controlled by small muscles to regulate gas exchange based on metabolic demands of the fly. Gas exchange of oxygen and carbon dioxide occurs directly through the tracheal system which innervate all organs and tissues of the fly. Nitric oxide (NO) in hypoxia is also required to modulate the tracheal system, akin to the vasodilation response in mammals. The fly tracheal (respiratory) system shares similar properties of plasticity with angiogenesis in the mammalian system (Romero et al., 2007). *Drosophila* tracheal terminal

branches have the capacity to sprout new projections towards hypoxic areas. This tracheal remodeling is achieved in part through induction of the FGF (Fibroblast growth factor)-FGFR (Fibroblast growth factor receptor) (Branchless *(bnl)*-Breathless *(btl)*) signaling cascade (Centanin et al., 2008). Under low oxygen conditions, this FGF-FGFR signaling is upregulated to increase tracheal branching to increase tissue oxygen supply. Pathological reduction in oxygen levels caused by gut infection or genetic induction of intestinal tumor-like overgrowths have also been shown to increase tracheation through the FGF pathway (Tamamouna et al., 2021). This tracheal remodeling can then provide the tumor-like overgrowths with a continued supply of oxygen, mimicking the process of increased angiogenesis in human tumors.

1.2.1.3 Organismal Responses to Low Oxygen

Fruit flies eat and lay eggs on the surface of in rotting fruit, a microenvironment rich in microorganisms and likely characterized by low oxygen. Hatched larvae then grow and develop in this environment by burrowing into and eating the yeast and microorganisms in the fermenting fruit, therefore being exposed to sustained periods of very low oxygen. The surface of fermenting fruit is also low in oxygen, which is where adult flies feed and lay their eggs (Barrows, 1907). Hence, *Drosophila* have likely evolved mechanisms to tolerate and adapt to this hypoxia. This ability to adapt to low oxygen levels makes *Drosophila* a useful model organism to study hypoxia tolerance mechanisms. Relatively few studies, however, have explored this although have shown how *Drosophila* are hypoxia tolerant, with the strength of these responses depending on the relevant life stage (Gorr et al., 2006). For example, embryos are the most hypoxia tolerant and can survive complete oxygen deprivation for several days (Teodoro & O'Farrell, 2003). At more moderate levels of hypoxia (5-10% O_2), *Drosophila* adults and larvae are viable although

larvae slow their growth, delay development to pupation and decrease their metabolic rate (Farzin et al., 2014; Lee et al., 2019; Callier et al., 2015; Callier & Nijhout, 2014; Kapali et al., 2022). Larvae and adult flies exposed to more extreme hypoxia (<1% O_2) enter a suspended animation-like state – adults cease feeding and, in the case of larvae, crawl away from their food, then become completely immobile. However, they can tolerate this level of hypoxia for up to ~24 hours. Studies have suggested that this behavioral response to hypoxia is a result of impaired neuronal cellular function (Haddad et al., 1997; Krishnan et al., 1997). Furthermore, in a comparative study in cultured fly and rat neurons, the fly neurons hyperpolarize in response to hypoxia and this phenomenon is thought to confer a protective effect (Gu & Haddad, 1999).

As indicated above, relatively few studies have explored the molecular and genetic mechanisms that control hypoxia responses in *Drosophila*. However, given the speed and versatility of fly genetics, conserved physiological mechanisms of growth, development and homeostasis, and their strong hypoxia tolerance, *Drosophila* provide an excellent model to study *in vivo* mechanisms controlling tissue and whole-body hypoxia responses. Below I will provide an overview of some key studies which have explored mechanisms of hypoxia tolerance in *Drosophila* and describe how they provide rationale for my thesis research.

1.2.2 Hypoxia Adaptation and Tolerance Mechanisms in Drosophila

1.2.2.1 Hypoxia-Inducible Factor (HIF)

Perhaps the best studied cellular adaptation to low oxygen conditions involves stabilization and induction of hypoxia-inducible factors (HIFs), which are conserved across metazoans (Rytkönen et al., 2011). *Drosophilae* have single homologs of both HIF-1α, called Sima, and HIF-1β, called

Tango (Erbel et al, 2003; Romero et al., 2007). They are regulated by hypoxia in the same way as their mammalian homologs – in hypoxia, Sima is stabilized and dimerizes with Tango to control gene expression (Figure 1). However, in comparison with our understanding of mammalian HIF-1α biology, very few studies have explored the role of Sima in *Drosophila.* HIF-1α/sima mutant larvae and adults are fully viable in normoxia but experience increased lethality in hypoxia, revealing the necessity of HIF for organismal hypoxia tolerance (Romero et al., 2007). One important adaptive response mediated by Sima that likely plays a role in these survival effects is hypoxia-induced increase in tracheal branching. This is mediated through Sima-dependent upregulation of the FGF-FGFR-like pathway responsible for tracheogenesis (Mortimer & Moberg, 2009). Induction of Sima in the larval hematopoetic organ, the lymph gland, also mediates production of macrophage-like cells in hypoxia (Mukherjee et al., 2011), while Sima in the larval fat body functions to suppress body growth via suppression of systemic insulin signaling, the main endocrine regulator of body growth (Hyun, 2013; Stern, 2003; Nässel et al., 2015). The one transcriptomic study that has looked for Sima target genes showed that Sima was important for controlling the expression of hundreds of genes, especially those involved in metabolism (Y. Li et al., 2013).

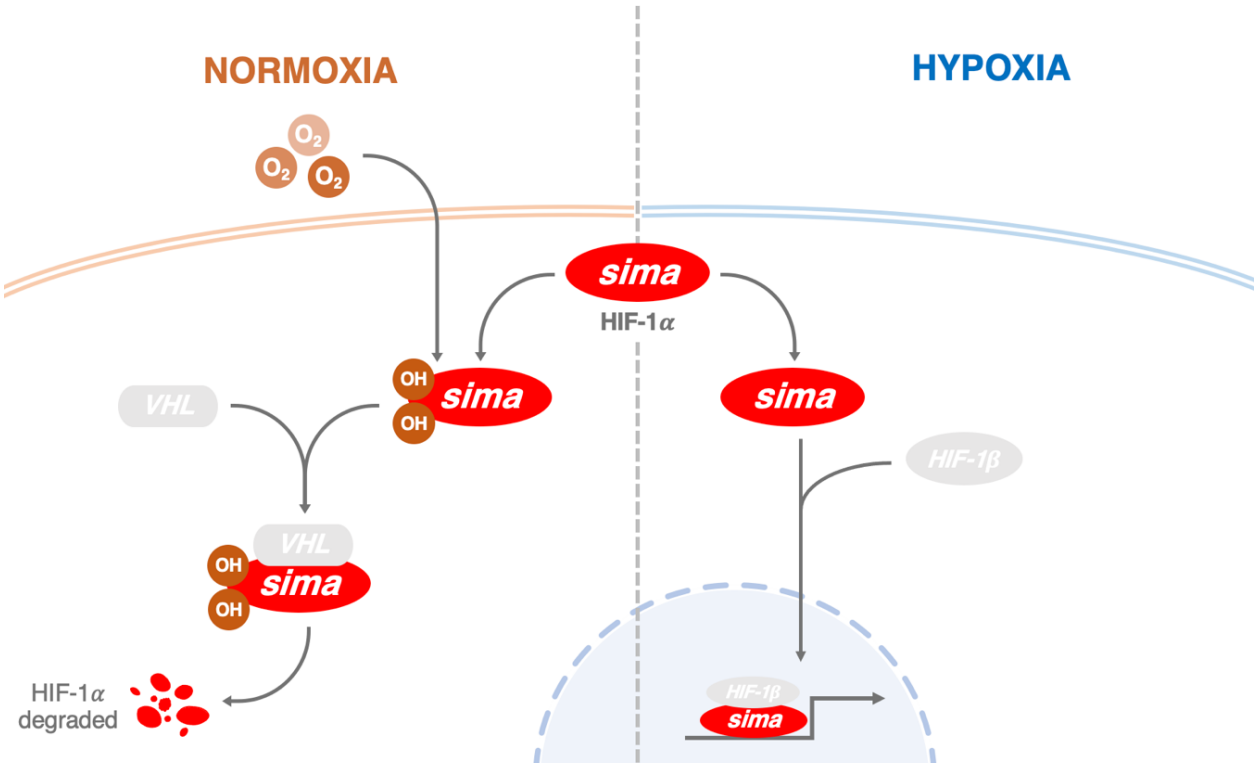

Figure 1. Regulation of HIF-1α/Sima in *Drosophila*. In normoxic conditions, HIF-1α/Sima is hydroxylated, ubiquitinated, and rapidly degraded. In hypoxia, HIF-1α/Sima is stabilized as it is no longer hydroxylated. HIF-1α/Sima and HIF-1β/Tango dimerize and translocate to the nucleus where they can promote transcription of target genes. *Created with BioRender.*

1.2.2.2 Nitric Oxide and cGMP Signaling

Organisms need to be able to sense their environmental conditions to maintain internal homeostasis. *Drosophila* sense fluctuations in the ambient air through O_2 and CO_2-sensing neurons. Specifically, oxygen levels are sensed by atypical soluble guanylyl cyclase (aSGC) subunits which respond to hypoxia by producing cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG) (Morton, 2004). Nitric oxide, another important hypoxia signal, is

detected by the conventional guanylyl cyclase family. Together, the integration of these NO/cGMP/PKG signals are important for the hypoxia escape behavioral response in larvae (Wingrove & O'Farrell, 1999; Vermehren-Schmaedick et al., 2010). This pathway also controls the recovery from hypoxia-induced suspended animation in low oxygen. In embryos, the NO/cGMP pathway also regulates anoxia-mediated changes in gene transcription and the cell cycle.

1.2.2.3 Insulin/TOR Kinase Signaling

The main regulators of larval body growth are the conserved insulin/PI3-kinase and TOR kinase growth signaling pathways (Grewal, 2009; Hietakangas & Cohen, 2009; Texada et al., 2020). A few recent papers have shown that hypoxia suppression of these two pathways is important for the reduced growth and viability in low oxygen seen in larvae. For example, Texada et al. (2019) demonstrated that HIF-1α/Sima in the *Drosophila* fat body, analogous to mammalian adipose and liver tissue, functions to inhibit insulin secretion from the brain to restrict growth. Our lab showed that hypoxia suppression of insulin upregulates activity of conserved transcription factor Forkhead Box-O (FOXO) to promote hypoxia survival in larvae and adults (Barretto et al., 2020). Another paper from our lab showed that the suppression of TOR in hypoxia is important for lipid metabolic remodeling and larval survival (Lee et al., 2019).

Independent of oxygen levels, insulin/TOR can also control translation and transcription of HIF-1α/Sima. Insulin has been shown to regulate HIF-1α protein expression through TOR-dependent translation pathways (Treins et al., 2002). Insulins and insulin-like growth factors (IGFs) can also increase HIF-dependent transcription, and this effect is also mediated by the TOR pathway

(Dekanty et al., 2005). TOR kinase can also directly stabilize HIF-1α by binding to the ODDD, preventing its degradation (Hudson et al., 2002). Therefore, insulin/TOR signaling has been shown to regulate HIF-1α at the level of both translation and transcription.

1.2.2.4 Co-Opting of Innate Immune Signaling

Two main branches of the innate immune response in *Drosophila* involve signaling through either the Toll or Imd (immune-deficient) pathways. Both lead to activation of the conserved nuclear factor kappa B (NF-κB) pathway and induction of antimicrobial peptides (AMPs). Canonically, the Toll and Imd pathways respond to presence of pathogenic gram-positive or gram-negative bacteria respectively (Myllymäki et al., 2014). Interestingly, two recent papers have shown that these pathways may be used in hypoxia to promote hypoxia survival. Banderra et al. (2014) showed that hypoxia induces components of Toll and Imd signaling pathways and NF-κB-dependent transcription. The upregulated Imd target genes were then shown to be important for hypoxia tolerance (Bandarra et al., 2014). A paper from our lab also demonstrated that the innate immune transcription factor of the Imd pathway, Relish/NF-κB, is upregulated in hypoxia by FOXO, and that this induction is needed for hypoxia tolerance (Barretto et al., 2020). These studies suggest that flies may co-opt immune signaling pathways to survive in low oxygen, perhaps by regulating the same type of physiological and metabolic tolerance responses seen following pathogenic infection.

A third branch of innate immunity in *Drosophila* involves signaling through the secreted cytokine family, the Unpaired ligands, and stimulation of JAK/STAT signaling. Interesting, an RNAseq analysis from our lab showed upregulation of Upds and JAK/STAT target genes in flies

exposed to 1% O_2 (Ding et al., 2022). These results raised the possibility that like Imd signaling, the Upd/JAK/STAT signaling pathway could be another innate immune response co-opted by flies in hypoxia. The central focus of my thesis has focused on investigating this possibility. Below I will provide an overview of the Upd/JAK/STAT pathway in flies and our current knowledge of its role as a stress and innate immune-induced signaling pathway.

1.3 Upd Cytokine/JAK/STAT Signaling in *Drosophila*

The JAnus Kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) pathway is an evolutionarily conserved signal transduction mechanism involved in regulating immune signaling and several developmental events. Studies first conducted in mammalian models have provided a canonical model in which cytokine binding to the cell surface receptor activates the tyrosine kinase JAK which associates with the intracellular domain of transmembrane cytokine receptors. JAKs then phosphorylate tyrosine on their associated receptors causing cytosolic STAT to bind the receptor complex. JAK-dependent phosphorylation activates the STAT molecules that homo- or heterodimerize before translocation to the nucleus. The STAT molecules are then able to bind specific DNA sequences and activate transcription (Darnell Jr., 1997; Zeidler et al., 2000) (Figure 2).

While vertebrates have numerous cytokines and growth factors which signal through the JAK/STAT pathway, *Drosophila* have three Unpaired (Upd) family genes located on the X-chromosome – Upd1, Upd2, Upd3 – which are the only ligands known to signal through the JAK/STAT pathway. The Upd ligands are secreted glycosylated proteins which bind to activate a

single type-I cytokine receptor Domeless *(dome),* homologous to the transmembrane protein receptor GP130 (Brown et al., 2001). Once an Upd ligand binds, the receptors dimerize and reciprocally transphosphorylase each other. In flies, Hopscotch *(hop)* is the 1177 amino acid non-receptor tyrosine kinase homologous to the human JAK2 (27% identity) (Binari & Perrimon, 1994). Vertebrates have many different receptors, compared to *Drosophila* where all three JAK/STAT ligands signal through one receptor. The STAT homolog STAT92E is homologous to human STAT5 (33% identity). STATs participate in gene regulation in response to extracellular signaling by polypeptides. Target genes of note include the negative regulator SOCS36E (Suppressor Of Cytokine Signaling at 36E) and the Turandot (*Tot*) stress genes. The fly SOCS36E has 68% identity to human SOCS-5 (Callus & Mathey-Prevot, 2002). *TotM* and *TotA* are JAK/STAT pathway responsive genes that are thought to play a role in *Drosophila* stress tolerance (Ekengren & Hultmark, 2001).

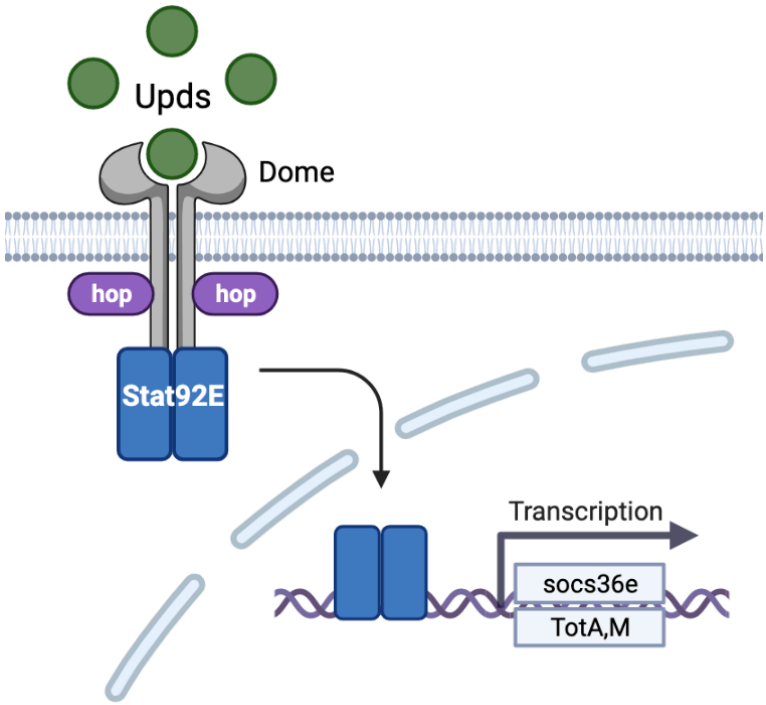

Figure 2. *Drosophila* JAK/STAT pathway. JAnus Kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) in *Drosophila* includes the singular cytokine receptor (*domeless*) which binds to all three Upd ligands – Upd1, Upd2 and Upd3. Upon receptor-ligand binding, the Janus Kinase (*hopscotch*) phosphorylates the receptor which causes cytosolic STAT92E to bind and hetero- or homodimerize before translocating to the nucleus. STAT92E binds certain DNA sequences and activates transcription of target genes such as *socs36e*, *TotA*, and *TotM. Created with BioRender.*

1.3.1 Functions of Upd3 in Stress Response

In response to different stresses, specific cells and tissues sense the stress, express and release Upds to act on local or distant tissues through JAK/STAT signaling to mediate adaptive responses to the stress. The specific Upd-producing and Upd-target cells and tissues differ based on the type of stress. In addition to immune responses and inflammation, Upd cytokines are

known to be involved in hematopoiesis, stem cell regeneration, metabolism, and development (Romão et al., 2021; Woodcock et al., 2015). Each of these events involve the JAK/STAT pathway regulating gene expression in a tissue- and stress-context dependent manner. The first ligand, Upd1, is largely needed during embryogenesis. Loss of Upd1 leads to segmentation defects and is embryonic lethal, suggesting it is required for several developmental processes (Nüsslein-Volhard & Wieschaus, 1980). During embryogenesis, Upd1 is also required for processes involving migration of primordial germ cells to the gonad (Brown et al., 2006). Upd2 and Upd3 mutants are, in contrast, viable under normal conditions. Upd2 is functionally homologous to the human Leptin hormone and controls insulin secretion to control body size (Rajan & Perrimon, 2012) while Upd3 is functionally analogous to human interleukin 6 (IL-6) (Oldefest et al., 2013). Expression levels of both Upd2 and Upd3 are low in basal conditions but are rapidly and strongly induced upon exposure to a variety of stresses. The increased expression of Upds allows them to be secreted and act in paracrine and endocrine manners on a range of tissues to mediate stress responses. Upd3 in particular shares evolutionarily conserved roles in mediating tissue to tissue signaling in response to immune challenge. Upd3 has been shown to play key roles in intestinal stem cell regeneration (Osman et al. 2012; Jiang et al., 2009), reproductive aging (Wang et al., 2014), glucose homeostasis and lifespan (Woodcock et al., 2015). Below I will provide examples of stressors that involve Upd2 and Upd3 signaling, with a focus on Upd3 since it is the primary focus of my thesis research.

1.3.1.1 Infection and Wounding Stress

The innate immune system plays a key role in protecting organisms from microbial infection and damage. The gut epithelial lining of animals is constantly being exposed to toxins, variable food

composition, mechanical or chemical stress, and pathogens. Several papers have shown that upon bacterial infection, damaged gut epithelial cells produce *upd3* which act locally on nearby stem cells to simulate their proliferation and differentiation to repair the damaged tissues (Osman et al., 2012; Buchon et al., 2009; von Frieling et al., 2020; Jiang et al., 2009). STAT92E also directly promotes transcription of antimicrobial peptides (AMPs) in response to infection (Buchon et al., 2009; Myllymäki et al., 2014). The Upds can also act long distance. One such study demonstrated that inflammatory cytokines from the gut can signal to the brain to control feeding responses after infection (Cai et al., 2021). Septic injury can also induce *upd3* in the *Drosophila* blood cells (hemocytes) and signal to the fat body for activation of stress-tolerance genes (Agaisse et al., 2003). In the case of parasitic wasp egg infection, circulating hemocytes release Upd2 and Upd3. This leads to STAT activation in somatic muscles that triggers lamellocyte differentiation and promotes egg encapsulation (Yang et al., 2015). Together, these studies emphasize how the JAK/STAT pathway plays an important positive role in immune response activation.

1.3.1.2 Tumor Stress

In humans, the immune system is thought to play an important role in mitigating the development of cancer, but tumor-derived inflammatory signals can also promote their growth. In *Drosophila* larvae, genetic induction of chromosomal instability in epithelial tissues causes tumor-like overgrowth. These tumors also cause an organismal delay in development and maturation by upregulating and secreting *upd3* which then suppresses the function of the prothoracic gland (PG), an endocrine organ that produces the maturation hormone ecdysone (Romão et al., 2021). Another paper showed that oncogenic Ras epithelial tumors upregulate

Upd cytokines, but in this case, they promoted macrophage proliferation and restricted tumor growth (Pastor-Pareja et al., 2008). In adult gut tumor models, *upd3* has been associated with cancer cachexia. Specifically, gut tumors release *upd3* and signal host tissues to produce ImpL2 (Insulin-like polypeptide binding protein), a negative regulator of insulin signaling, causing host tissue wasting and insulin resistance (Ding et al., 2021). Moreover, increased JAK/STAT signaling is associated with increased invasiveness and likelihood of metastasis (Rattigan et al., 2010; Dorritie et al., 2014; Amoyel et al., 2014). Finally, a direct link with cancer is seen in dominant gain-of-function allele, Tumorous lethal ($hop^{Tum\text{-}l}$) mutants, which develop malignant neoplasms in the *Drosophila* blood cells (Harrison et al., 1995).

1.3.1.3 Nutrient Stress

In both mammals and flies, cytokines play a conserved role in nutrient sensing and metabolic homeostasis. In flies, this regulation has been shown to be important in situations of both nutrient starvation and nutrient excess. For example, in larvae, proper nutrient availability promotes Upd2 production from the *Drosophila* fat body (analogous to the mammalian liver and adipose tissue) which then acts on the brain to promote release of *Drosophila* insulin-like peptides (Dilps) which are necessary for maintenance of glucose homeostasis and promoting growth (Rajan & Perrimon, 2012). However, upon starvation the Upd2 that mediates gut-to-brain signaling is suppressed and insulin release is blocked. In a pathological setting of nutrient excess, it has been demonstrated that flies fed a high fat diet upregulate macrophage derived Upd3 which causes insulin resistance and reduction in lifespan (Woodcock et al., 2015). These findings in flies are akin those in murine models showing a role for macrophage release of IL-6 in mediating insulin resistance (Han et al., 2013). Another paper showed that high fat diet induces Upd3 in gut

to promote stem cell proliferation, and that this is dependent on the microbiome and JNK signaling (von Frieling et al., 2020).

1.4 Regulation of Upd3 Expression in *Drosophila*

Although the Upd producing and target tissues differ based on the stress context, they share similar mechanisms of Upd induction. I will describe these pathways, their induction, and the mechanism of subsequent Upd3 induction below.

1.4.1 Reactive Oxygen Species (ROS)-Dependent Activation of JNK and p38 Signaling

The main signaling cascade which is known to induce Upd3 under stress conditions is through ROS/JNK/p38. Reactive oxygen species (ROS) are a family of highly reactive molecules which form from diatomic oxygen (Murphy et al., 2011). Each of the species have different properties relating to reactive potential, mechanisms of degradation and diffusion in biological organisms. The principal production of ROS in the mitochondria has important implications in organismal homeostasis and cellular stress responses (Shadel & Horvath, 2015; Hamanaka & Chandel, 2010). Endogenous ROS are produced by the cytosolic enzymes NOX (NADPH oxidase)/DUOX (Dual oxidase) first discovered to be important for the host defense in response to microbes (Lambeth, 2004). Given the differences in the chemical properties of the various species, antioxidants act on specific ROS (Krumova & Cosa, 2016). For instance, superoxide dismutase (Sod) selectively targets superoxide anions and turns them into the slightly less harmful hydrogen peroxide to prevent conversion to the more reactive peroxynitrate. Another antioxidant enzyme, catalase (Cat), converts hydrogen peroxide to water and molecular oxygen. In

Drosophila, physical injury triggers a series of events including a burst of ROS at the injury site to attract immune cells (Moreira et al., 2010). Endogenous ROS have important signaling roles, but excess ROS can cause oxidative stress. In infection, production of hydrogen peroxide through DUOX in hemocytes is necessary for *upd3* induction and is essential for survival under infection and wounding (Chakrabarti & Visweswariah, 2020). Another study elucidated a role for local paracrine ROS signaling in regulating *upd3* expression from pericardial cells to maintain healthy heart function and life span (Gera et al., 2022).

Two main effectors of ROS in *Drosophila* are the JNK and p38 signaling pathways. c-Jun N-terminal kinases (JNKs) regulate cellular responses to radiation, DNA damage, heat, pathogens, reactive oxygen species (ROS), and inflammatory cytokines. In *Drosophila*, the conserved JNK pathway is required to regulate normal morphological processes during development in addition to mounting innate immune responses. JNKs phosphorylate and activate the transcription factor c-Jun, a component of the activator protein (AP-1) complex that regulates gene expression (Pulverer et al., 1991). A single JNK exists in *Drosophila*, called Basket *(bsk)*. Other conserved pathway components include AP-1 complex components Jun-related antigen *(Jra)* and Kayak *(kay)*, homologous to JUN and FOS respectively (La Marca & Richardson, 2020). In the *Drosophila* intestine, JNK-mediated stress signaling triggers enterocytes to produce Upds which promote stem cell division and maintain gut homeostasis (Jiang et al., 2009). In a model of cardiac aging, *Drosophila* oenocytes (hepatocyte-like cells) were shown to induce *upd3* through JNK, suggesting increased *upd3* in oenocytes contributes to age-dependent cardiac dysfunction (Huang et al., 2020). Lipid-rich diets induce *upd3* from macrophages, also through JNK, resulting in insulin resistance and lifespan reduction (Woodcock et al., 2015).

p38 is an evolutionarily conserved signaling mechanism involved in regulating apoptosis, cell fate determination, immune and stress response (Ono & Han, 2000; Jia et al., 2007). Impaired p38 signaling has multiple implications in human disease, such as cancer, heart disease, arthritis, and neurodegenerative diseases. Three orthologs of p38 exist in *Drosophila* – p38a, p38b, p38c. Each differs in their context-dependent functional importance while also sharing some functional redundancy (Chen et al., 2010). Recent work in *Drosophila* have demonstrated a link between the ROS/p38/JNK signaling axis and induction of *upd3* in response to cell death to promote imaginal disc regeneration (Santabárbara-Ruiz et al., 2015). The authors demonstrated that ROS-dependent activation of both JNK and p38 are required to promote *upd3* expression upon tissue injury. In another study, p38-dependent MAPK pathways were implicated in *upd3* regulation in the gut upon infection (Houtz et al., 2017).

1.4.2 Src-Kinase Signaling

Src family tyrosine kinases act as intermediates for many cellular processes such as cell proliferation, survival and differentiation, and cytoskeletal rearrangement. Src-kinases can also activate MAPK signaling pathways. For example, Src-dependent tumor-like overgrowth was shown to require JNK and STAT function but attenuating STAT function could cause the tumorous cells to undergo apoptosis instead (Read et al., 2004). It was also found that the Src was required together with TGF-β and Hippo signaling for *upd3* upregulation and intestinal stem cell renewal upon bacterial infection (Houtz et al., 2017). Src kinases can also act downstream of ROS during wound repair to promote polarization of the extracellular matrix (Hunter et al., 2018; Juarez et al., 2011).

1.4.3 Hippo Signaling

The highly conserved Hippo signaling pathway is required for normal development. Its activation suppresses growth by inactivation of Yki (YAP in vertebrates), a transcriptional coactivator, and its deregulation has been associated with number of cancers (Reddy & Irvine, 2008; Badouel et al., 2009). The pathway is named after the Sterile 20-like kinase Hippo (*hpo*) which forms a complex with scaffolding protein Salvadore (*sav*) to phosphorylate kinase Warts (*wts*). Phosphorylated Wts is then able to associate with the mob as tumor-suppressor (*Mts*) protein to phosphorylate the transcriptional coactivator Yorkie (*yki*). This phosphorylation leads to cytosolic retention of Yki, thus preventing transcription of Yki target genes that function to promote cell proliferation and prevent apoptosis (Zhao et al., 2007; Badouel et al., 2009). Studies have shown that the Hippo pathway is required for ISC proliferation and gut homeostasis in response to damage via Yki activation which upregulates *upd3* expression (Li et al., 2014; Staley & Irvine, 2010; Shaw et al., 2010). This upregulation of Upd3 by Yki is likely mediated through direct DNA-binding of the Yki partner, Scalloped (*Sd*), homologous to mammalian Tead, to the Upd3 promoter since Scalloped was identified as binding to the *upd3* promoter through a yeast one-hybrid assay. An Upd3 promoter-GFP reporter containing Scalloped binding sites also showed strong induction in the intestine upon stress (Houtz et al., 2017).

1.4.4 Transforming Growth Factor Beta (TGF-β)/Smad Signaling

The conserved TGF-β signaling pathway exists in *Drosophila* in a simplified form. As in mammals, there are two branches of the *Drosophila* TGF-β pathway which are activated by different ligands, activins, and bone morphogen proteins (BMPs). The receptors of this pathway are transmembrane serine/threonine protein kinases consisting of heterodimeric type I and type II

subunits. Activation of the signaling cascade via ligand-receptor binding leads to phosphorylation of the type I receptor by the constitutively active type II receptor. This event triggers phosphorylation of a receptor-activated Smad (R-Smad) substrate –Mad. The R-Smad can then bind to the *Drosophila* common Smad (co-Smad), Med (*medea)*, and form a complex which is able to translocate to the nucleus and promote transcription of target genes (Upadhyay et al., 2017). The TGF-β pathway has been previously implicated in a main regulator of intestinal homeostasis by regulation of ISC proliferation and quiescence and EC differentiation and protection (H. Li et al., 2013; Guo et al., 2013; Tian & Jiang, 2014). However only study has showed evidence that Mad was necessary for *upd3* induction during pathogenic microbe ingestion (Houtz et al., 2017). There is also evidence to show crosstalk between the BMP and Hippo pathway, as Yki has been shown to be able to bind Mad and control *bantam* transcription and thus integrate the two growth control pathways (Oh & Irvine, 2010). Together, TGF-β, Hippo and Src pathways all play a role in enterocyte (EC)-specific induction of *upd3.*

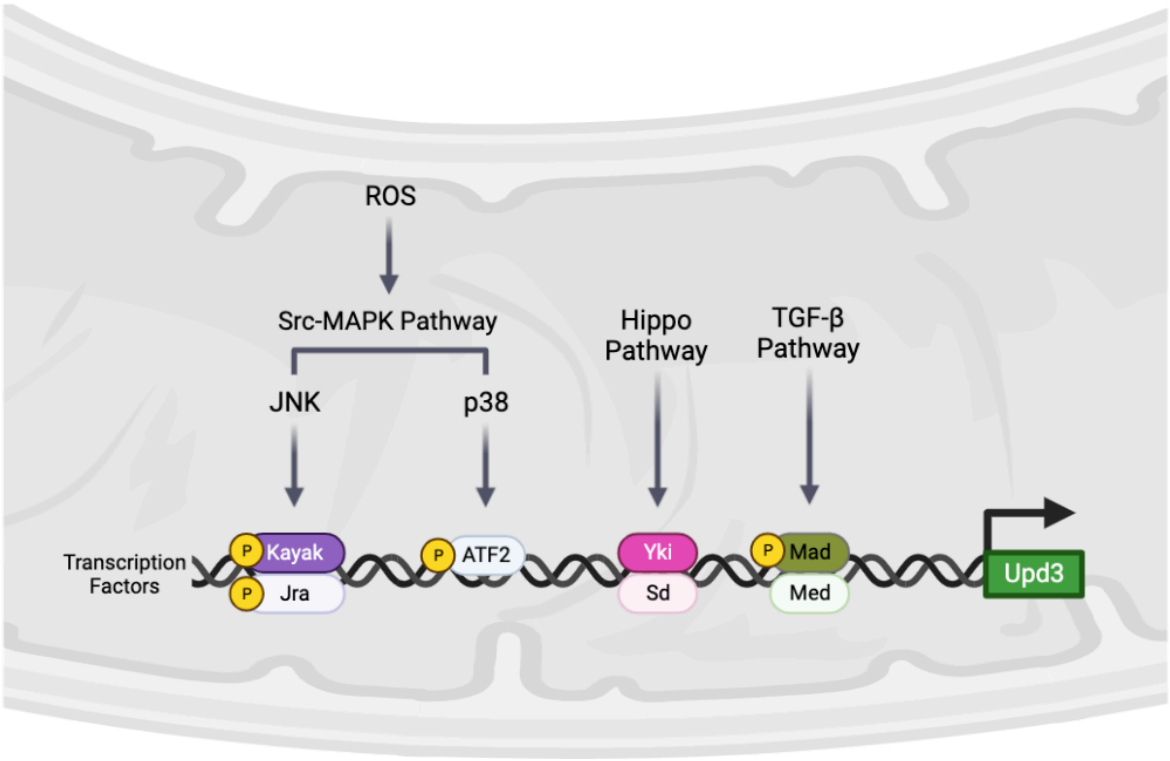

Figure 3. Regulators of *upd3* transcription in the *Drosophila* intestine. In response to external stimuli, *upd3* can be induced through integration of various signaling pathways, including but not limited to: ROS-Src-MAPK [JNK (p-Kayak, p-Jra), p38 (p-ATF2)]. Hippo (Yki, Sd), and TGF-**β** (p-Mad, Med). *Created with BioRender.*

1.5 Non-autonomous Responses to Stress in *Drosophila*

Although almost all tissues have some capacity to sense environmental challenges such as low oxygen, my work focuses on two tissues – the *Drosophila* fat body and gut. Below I will discuss examples of their role in organismal physiology.

1.5.1 Fat Body

The *Drosophila* fat body is a tissue functionally analogous to mammalian hepatic and adipose tissue. It acts as one of the main mediators of non-autonomous signaling, storing nutrients, and coordinating endocrine signaling in response to various external signals – namely nutrient sensing (Britton & Edgar, 1998; Colombani et al., 2003). In response to nutrient deprivation, autophagy in the larval fat body helps animals to maintain hemolymph nutrient levels (Scott et al., 2004; Rusten et al., 2004). While most studies have looked at the fat body's role in nutrient sensing, it has also been shown to play essential roles in sensing other environmental cues. For instance, it had been shown that the larval fat body directly senses low oxygen conditions and releases factors that suppress growth in hypoxia via suppression of insulin signaling (Texada et al., 2019). At the same time, the HIF-1α/Sima in the fat body releases FGF-like factor Branchless (*bnl)* which promotes tracheation towards the oxygen-deficient areas (Texada et al., 2019).

1.5.2 Gut

Animals often face insults to their intestinal tract, which requires coordination of regenerative processes. Perhaps the best studied of these insults in *Drosophila* is in the case of enteric infection. In this example, damage to the intestine caused by the infection triggers ISC mitosis to replace the damaged cells (Jiang et al., 2009; Buchon et al., 2009). Specifically, activation of the Upd3/JAK/STAT pathway is required for ISC differentiation into mature ECs. More recent studies have shed light on a role for tracheal oxygen supply as the mediator of gut homeostasis. Such studies show that intestinal ROS are produced by both epithelial tumors and damaged ECs during infection. ROS-dependent activation of Sima then increases tracheal branching and oxygen availability to the intestine, which is required for ISC mitosis (Tamamouna et al., 2021; Perochon et al., 2021).

1.6 Objectives and Aims

Immune signaling is necessary for conferring hypoxia tolerance in *Drosophila.* In other stress contexts, Upd3 is known to act as a long-range signaling molecule to coordinate tissue-to-tissue communication with its effect varying based on the producing and target tissues. However, the role of Upd3 in hypoxia remains unknown. I aim to explore this in my thesis with the following objectives:

1. Explore whether Upd3 is needed for hypoxia tolerance.
2. Identify in which tissue(s) Upd3 is important for hypoxia tolerance.
3. Identify how Upd3 alters gene expression to promote adult hypoxia tolerance.

Given that cytokine signaling is similar in flies and mammals, my discoveries may point to conserved regulation of hypoxia responses in both normal and pathological conditions.

CHAPTER TWO: MATERIALS & METHODS

2.1 Drosophila

2.1.1 Drosophila Stocks

Descriptions of stocks used throughout this investigation are listed in Tables 1 and 2. Further descriptions of these mutations and balancer chromosomes may be found in FlyBase (http://flybase.org/).

Name	Genotype	Source/Reference
w^{1118}	*w^{1118}; +; +*	BDSC
GD	*w^{1118}; +; +*	VDRC (60000)
KK	*y,w^{1118}; +; +*	VDRC (60100)
Attp2	*yv; +; +*	BDSC
daGal4	*w^{1118}; +; da-gal4*	BDSC (8641)
r4Gal4	*w^{1118}; +; r4-gal4*	BDSC (33832)
mexGal4	*w^{1118}; mex-gal4; +*	Phillips & Thomas, 2006
Myo1AGal4	*w*; Myo1A-gal4; +*	Savraj Grewal Lab
da-GSG	*w^{1118}; da-gal4 GeneSwitch; +*	Savraj Grewal Lab
UAS-CatA	*w^{1}; UAS-CatA; +*	BDSC (24621)
UAS-Sod1	*w^{1}; UAS-Sod1; +*	BDSC (24750)
UAS-sima RNAi	*+; UAS-sima RNAi; +*	VDRC (106187/KK)
UAS-p38b RNAi	*y^{1}v^{1}; +; UAS-p38b RNAi*	BDSC (29405)
UAS-Kayak RNAi	*+; +; UAS-Kayak RNAi*	VDRC (6212/GD)

UAS-bskDN	*w^{1118}, UAS-bskDN; +; +*	BDSC (6409)
UAS-upd3 RNAi	*+; +; UAS-upd3 RNAi*	VDRC (27136/GD)
UAS-hopIR	*$y^{1}v^{1}$; +; UAS-hop RNAi*	BDSC (32966)
UAS-HexA RNAi	*y^{1}sc*$v^{1}sev^{21}$; +; UAS-HexA RNAi*	BDSC (35155)
UAS-mad RNAi	*$y^{1}v^{1}$; +; UAS-mad RNAi*	BDSC (31316)
UAS-tkv RNAi	*$y^{1}v^{1}$; UAS-tkv RNAi; +*	BDSC (40937)
UAS-upd3	*w^{1118}; UAS-upd3; +*	Bruno Lemaitre Lan (Shin et al., 2020)
UAS-yki RNAi	*$y^{1}v^{1}$; +; UAS-yki RNAi*	BDSC (34067)
upd3*Δ*; UAS-sima RNAi	*w*, upd3*Δ*; UAS-sima RNAi; +*	This work
***upd3*Δ**	*w*, upd3*Δ*; +; +*	BDSC (55728)
upd3Gal4, UAS-GFP/Cyo	*+; upd3-gal4, UAS-GFP/Cyo; +*	Savraj Grewal Lab (Agaisse et al., 2003)

Table 1. *Drosophila* stocks used throughout this work.

*w** and *sc** indicate the precise allele of *w* and *sc* are unknown. 'BDSC' refers to the Bloomington *Drosophila* Stock Center. 'VDRC' refers to the Vienna *Drosophila* Resource Center.

2.1.2 Control Strains

Throughout this work, w^{1118} was used a wild-type strain. In Gal4-UAS crosses, w^{1118} was used as the control strain for w^{1118} background flies, *GD* or *KK* were used as a control strain for VDRC lines, and *Attp2* was used as a control strain for BDSC TRiP lines.

2.1.3 Drosophila Husbandry

Drosophila stocks were maintained at 25ºC or 18ºC. All stocks were maintained on media comprised of 100g *Drosophila* Type II agar, 1200g cornmeal, 490mg Torula yeast, 450g sugar, 1240g D-glucose, 160mL acid mixture (propionic and phosphoric acid) in 20L water. Genetic crosses were established by mating virgin females with males. All crosses and progeny were maintained at 25ºC.

2.1.4 Egg Collection

Drosophila were allowed to lay eggs on grape juice agar plates with yeast paste as a food source. For adult experiments, egg-laying on each agar plate occurred overnight and hatched larvae were transferred to food vials 24 hours after egg laying (AEL) in groups of 50 larvae. Grape juice agar was prepared from 250mL grape juice concentrate (Minute Maid brand), 25g sugar, 22.5g agar, 750mL MilliQ H_2O, and 1.5g nipagin (methyl 4-hydroxy benzoate, Sigma).

2.1.5 Gene Expression Systems

2.1.5.1 Gal4-UAS system

The Gal4/Upstream Activator Sequence (UAS) system is used to knockdown and overexpress genes of interest in specific tissues of interest (Brand & Perrimon, 1993). Use in *Drosophila* was originally adapted from yeast in which transcriptional regulator Gal4 induces transcription of a gene of interest under UAS control (Duffy, 2002). The two components of the system, the Gal4 driver and UAS responder, are maintained as separate parental lines. Genetic manipulation is then achieved by crossing these lines to produce progeny which contain both. This system is especially useful for manipulation of genes that affect reproductive viability Gal4 is under the

control of different promoter and enhancer sequences to drive UAS-mediated gene expression in a variety of different tissues and cell types. Overexpression is achieved by placing a UAS sequence upstream of the target gene. Additionally, this system can also be used to knockdown genes by cloning the UAS sequence upstream of an inverted repeat. This forms a double stranded RNA for RNAi mediated loss of function (Perrimon et al., 2010). To control for genetic background effects, control animals are obtained by crossing the relevant Gal4 line to flies with the same background as the UAS transgene being used.

2.1.5.2 GeneSwitch Gal4-UAS

A variation of this system, the Gal4-GeneSwitch/UAS system is another tool for inducible gene expression through mifepristone (RU486) steroid-feeding (Roman et al., 2001; Osterwalder et al., 2001). This Gene-Switch fusion protein system contains the Gal4 DNA-binding domain fused to the ligand-binding domain of the human progesterone receptor and the activation domain of p65. In the presence of antiprogesterone RU486, Gene-Switch changes confirmation and translocates to the nucleus where it binds a UAS to provide inducible target gene activation. The benefit of this modified system is that it allows for restricted overexpression or knockdown of genes specifically to the adult stage, thus bypassing any potential lethal effects caused by gene knockdown or overexpression in pre-adult stages. This system also allows for control of absolute gene expression level depending on the concentration of RU486 and the duration of feeding. This system also reduces the potential confound of different genetic backgrounds. Control flies are fed a vehicle control instead of RU486, and therefore do not express the gene of interest.

Driver	Major Tissue	Expression pattern
daGal4	Organism | Ubiquitous	Drives ubiquitous expression in embryos, larvae, and adults.
daGSG	Organism | Ubiquitous	Drives mifepristone-inducible expression ubiquitously.
r4Gal4	Fat body	Drives expression in the fat body in a sex-nonspecific matter from late embryo through adult stages.
mexGal4	Midgut	Drives expression in enterocytes and midgut.
Myo1AGal4	Enterocyte Midgut	Drives expression in enterocytes and midgut.

Table 2. Expression patterns of Gal4 drivers.

2.2 Hypoxia Experiments

2.2.1 Hypoxia Exposure

For all hypoxia experiments, *Drosophila* were placed into a chamber with a constant flow of 1% oxygen (1% oxygen/99% nitrogen) at room temperature. The flow rate was controlled using an Aalborg model P gas flow meter.

2.2.2 Adult Hypoxia Survival

Adult hypoxia survival assays were conducted on *Drosophila* reared in food vials of 50 larvae or bottles of approximately 200 larvae. *Drosophila* were maintained in an incubator at 25ºC, allowed to eclose and mate for 2-3 days. Adult flies were then anaesthetized with CO_2 and sorted into vials of 15-25 males or females. Adults were allowed to recover from anaesthetization in normoxia for 5 days, during which they were flipped to new vials of food regularly. Females were placed in 1% O_2 hypoxia for 24-26 hours, and 1% O_2 hypoxia males were exposed to 16-18 hours. The reason for the difference in exposure time between males and females is owed to the

fact that males are inherently less tolerant to hypoxia than females. Therefore, the duration of exposure was determined based on how long it took for the control genotype survival to reach 50-80%. This range allowed for observation of either increase or decrease in survival of the experimental flies. Adults were allowed to recover from hypoxia exposure for at least 48 hours before the number of surviving flies was counted.

2.3 Visualization of GFP Reporters

2.3.1 Adult Dissection

Age-matched adult female flies were rinsed with 75% EtOH in a Petri dish. For dissection under a light microscope, flies were transferred in a watch glass containing ice-cold 1xPBS. Using fine forceps, adult tissues were isolated.

2.3.2 Fixation and Nuclei Staining

Fixation and nuclei staining were performed at room temperature unless otherwise specified. Following dissection, tissues were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in 1xPBS for 20 minutes. Hoechst 33342 was added (1:10 000 dilution) for 10 minutes. Tissues were washed three times in 1xPBS for 15 minutes prior to mounting.

2.3.3 Visualization of Dissected Tissue

Tissues were mounted on glass slides with cover slips, using mounting media Vectashield (Vector Laboratories Inc.). Cover slips were sealed to slides using clear nail polish. Slides were then visualized on a Zeiss Observer Z1 microscope or on a Zeiss LSM 880 Laser confocal

microscope using 10x and 20x objectives and Axiovision software. For consistency, exposure times for each experiment were based upon the control sample.

2.4 Feeding Treatments

2.4.1 Starvation

7-day-old adult flies were placed in vials (25 per vial) containing either 0.4% agar/PBS (starved) or standard *Drosophila* media (control).

2.4.2 Antibiotics

Adults were placed in vials containing an antibiotic cocktail (100µg/mL Ampicillin, 100µg/mL Neomycin, 100µg/mL Metronidazole, and 40µg/mL Vancomycin) or vehicle control ($1H_2O$: 5.5EtOH) for 5-7 days. Absence or presence of the gut microbiome was verified by streaking fly lysates on LB broth plates:

Prepared and autoclaved:

LB broth: 0.725g LB base (BD Difco) + 50mL MilliQ water

LB agar plates: 15.5g LB base + 1000mL MilliQ water + 15g Select Agar (Invitrogen)

LB agar plates were poured in sterile 100x15mm Petri dishes (VWR) and allowed to set overnight.

To sterilize the outer cuticle of flies, flies were washed with 75% EtOH and washed with sterile MilliQ water. Samples were then homogenized in previously autoclaved LB broth using pestles and pestle motor (VWR). Debris was allowed to settle to bottom of tubes before preparing 10-fold sample dilutions (10^{-1}, 10^{-2}, 10^{-3}). Undiluted and 10^{-3} diluted lysates were plated for each

sample using a sterilized single-use cell spreader. Plates were placed at 37ºC for 1-2 days, then monitored for colony growth.

2.4.3 RU486 Treatment

Adults were placed in vials containing standard *Drosophila* media supplemented with 100µM RU486 or vehicle control for 5-7 days.

2.5 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

2.5.1 Sample Collection

Drosophila adults were reared under normal conditions and anaesthetized using CO_2 after exposure to normoxia or 16 hours 1% O_2 hypoxia. Flies were then transferred into 1.5mL microtubes in groups of 5 before snap freezing on dry ice. For tissue-specific samples, adult females were taken out of 1% O_2 hypoxia or normoxia and immediately washed in 75% EtOH to remove hydrophobic outer layer. Groups of 5-10 females were transferred into glass dissection dish containing cold 1xPBS using fine forceps. Tissues were dissected and immediately transferred to 1.5mL microtubes containing 500mL TRIzol reagent (Invitrogen) before snap freezing on dry ice. All samples were stored at -80ºC until RNA isolation.

2.5.2 RNA Isolation

Whole animal samples: 250µL of TRIzol was added to 1.5mL microtubes containing samples. Samples were then homogenized using pestles and pestle motor (VWR). An additional 250µL of TRIzol was added to tubes.

Tissue samples: Samples in 1.5mL microtubes containing 500µL of TRIzol were homogenized using pestles and pestle motor (VWR).

RNA was isolated according to manufacturer's instructions (Invitrogen; 15596-018). 50µL of RNase/DNase-free UltraPure water was used to resuspend RNA pellets from tissue samples and 200µL was used to resuspend whole animal RNA pellets. Suspended RNA was stored at -80ºC for long-term storage.

2.5.3 DNase Treatment and cDNA Synthesis

RNA quality was measured using a NanoDrop OneC Spectrophotometer. Within any given experiment, equal volumes of RNA were used for cDNA synthesis and normalization of gene expression results were completed using a standard housekeeping gene. RNA samples were DNase treated following manufacturer's instructions (Ambion; 2238 G). Reverse transcription was achieved using SuperScript III (Invitrogen; 18080044). For whole animal and tissue samples, cDNA was synthesized using the following volumes:

Whole animal samples: 4µL of DNase-treated RNA was added to new 200µL PCR strip tubes. 1µL dNTPs (Invitrogen), 1µL Random Primers (Invitrogen) and 6µL RNase/DNase-free water (UltraPure, Invitrogen) was added to each tube.

Tissue samples: 10µL of DNase-treated RNA was added to new 200µL PCR strip tubes. 1µL dNTPs (Invitrogen) and 1µL Random Primers (Invitrogen) was added to each tube.

2.5.4 qRT-PCR

The generated cDNA was used as a template for qRT-PCR reactions (QuantStudio 6 RT-PCR system, Applied Biosystems) using the primer pairs defined in Table 2 and SYBR Green reagents. Per each reaction, the following master mix volumes were used:

2µL cDNA

0.3µL each forward and reverse primer in 4µL RNase/DNase-free water

0.26µL dNTPs

0.39µL $MgCl_2$

1.3µL 10x PCR buffer (-$MgCl_2$)

0.13µL SYBR Green

0.26µL ROX Reference Dye

0.05µL Platinum Taq DNA Polymerase

4.61µL RNase/DNase-free water

qRT-PCR amplification protocol followed a protocol for standard curve, standard ramp speed, and SYBR Green reagents. Amplification was achieved through the following protocol:

1. Hold: 50ºC for 2 minutes
2. Initial denaturation: 95ºC for 10 minutes
3. Denaturation: 95ºC for 15 seconds
4. Annealing: 60ºC for 1 minute

Steps 1-4 repeated for 40 cycles.

2.5.5 Data Analysis

Data analysis for qRT-PCR was completed using the comparative C_T method ($2^{-\Delta\Delta CT}$). The following equation was used to perform data analysis:

ΔC_T (sample) = C_T target gene - C_T reference gene

ΔC_T (control) = C_T target gene - C_T reference gene

Note: Reference (housekeeping) genes denoted in Table 3 by an asterik (*). In each qRT-PCR run, multiple reference genes were run and selected only if expression was not affected by genotype or experimental condition.

The ΔC_T values for each target gene of interest were then normalized to an experimental control:

$\Delta\Delta C_T = \Delta C_T$ (sample) - ΔC_T (control)

Normalized target gene expression levels were used to calculate relative fold change for each gene of interest = $2^{-\Delta\Delta CT}$

2.5.6 Primer Design

Primer Name	Forward Sequence	Reverse Sequence
HexA	CACATCCCAAGGCGAATGTCA	CGGGACTCCATTTGAAAATCGTT
Ldh	AGATCCTGACTCCCACCGAA	GCCTGGACATCGGACATGAT
Upd2	TGCGGAACATCACGATGAGCGAAT	TCTTCTGCTGATCCTTGCGGAACT
Upd3	ACAAGGCCAGGATCACCACCAAT	TGTACAGCAGGTTGGTCAGGTTGA
Socs36e	AGTGCTTTACTGCTGCGACT	TCGTCGAGTATTGCGAAGT
TotA	TCAACTGCTCTTATGTGCTTTGC	CTCACGATCTTCGTCGGAATAG
TotM	TTGAGCTGCCTTATGGTCTTCT	CGCTGTTTTTCTGTGACGAACT
bnl	TGCCCTATCACAGAGTTGC	ACCTACACGAACGCCATCAC

*Act5C	GAGCGCGGTTACTCTTTCAC	ACTTCTCCAACGAGGAGCTG
*bTub	TGGAGAACACAGACGAGACG	CGAGACCAGGTGGTTCAAGT
*5S rRNA	ACGACCATACCACGCTGAAT	AGCGGTCCCCCATCTAAGTA
*18S rRNA	ATGCACCACCACCCATAGAT	CCTGCGGCTTAATTTGACTC
*eIF-2α	TCTTCGATGAGTGCAACCTG	CCTCGTAACCGTAGCAGGAG
*RpS24	CCATGTACAAGGTCACCCC	CGGTACTTGGGCTCGAACTT

Table 3. Primers used for cDNA amplification.

All primers listed in 5'- 3'direction.

2.6 Statistics

For all experiments, error bars represent Standard Error of the Mean (SEM), and p-values are the result of unpaired *t*-test or two-way Analysis of Variance (ANOVA) followed by Student's *t*-test using GraphPad Prism (v.9). $p<0.05$ was considered significantly significant and indicated by an asterisk (*).

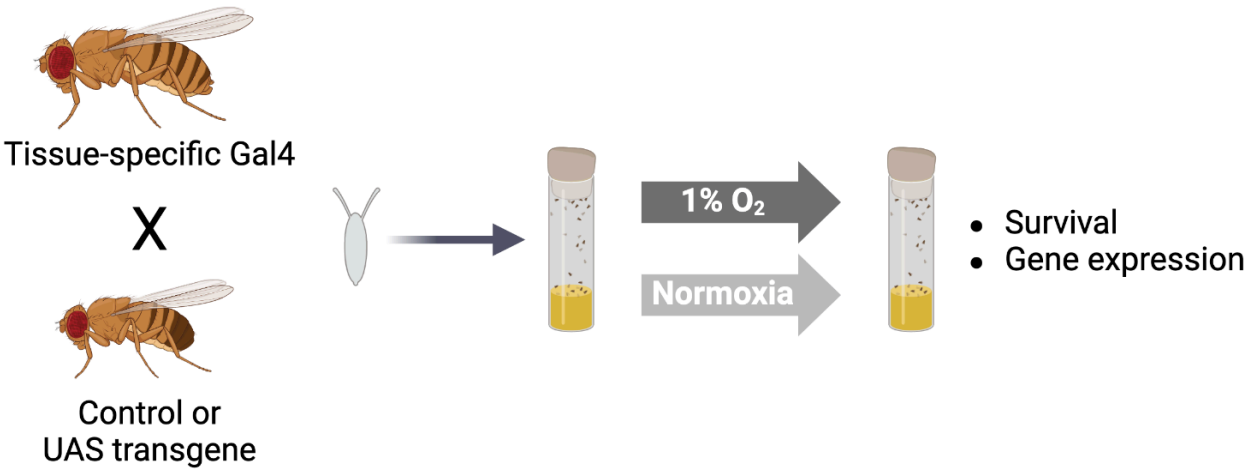

Figure 4. Experimental outline for hypoxia experiments. Tissue-specific Gal4 driver lines (see Table 2 for Gal4 driver expression patterns) were crossed to a UAS transgenic line and allowed to lay eggs on grape plates overnight. 24 hours after egg laying (AEL), larvae were transferred to vials, then allowed to pupate and eclose. Adult flies were mated for 2 days, then female and males were separated in vials. Once animals were 7 days old, animals were exposed to either 1% O_2 or maintained in normoxia. Number of dead flies in each vial were counted (survival) or collected for qRT-PCR (gene expression). *Created with BioRender.*

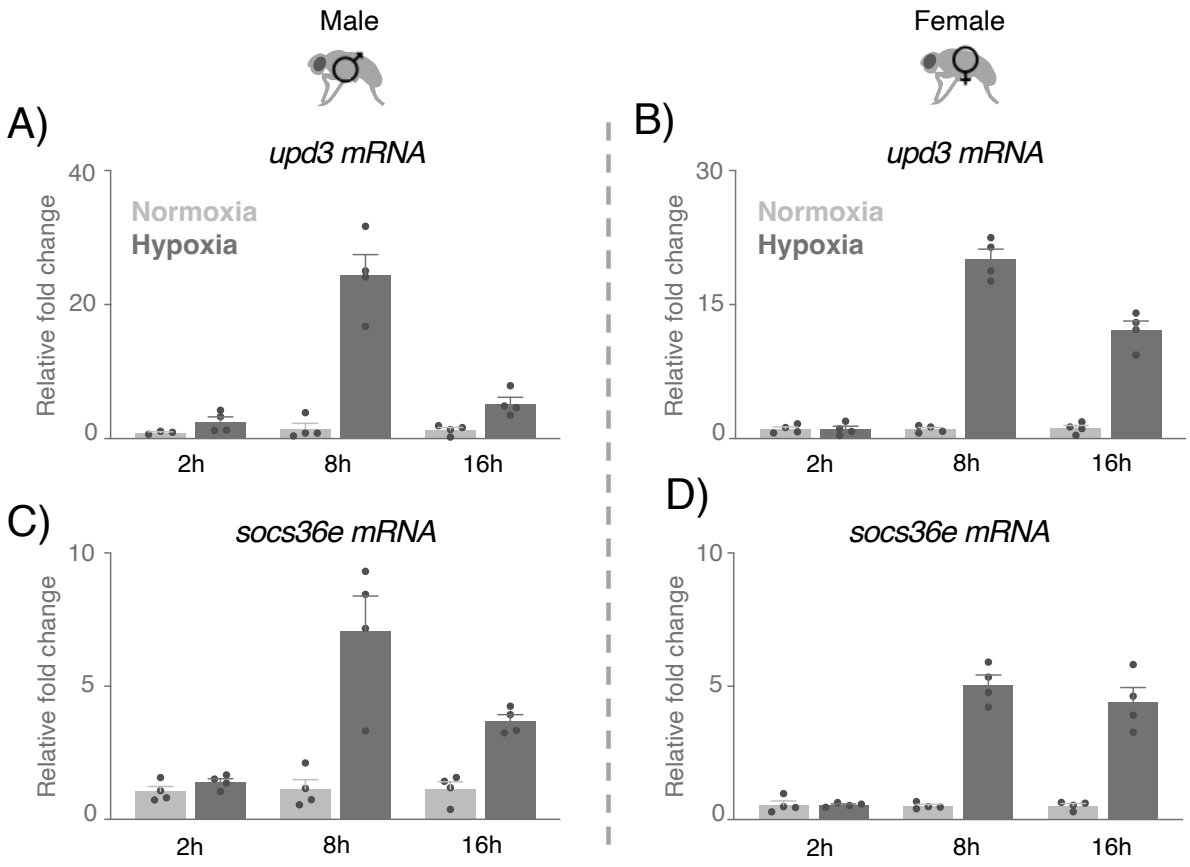

Figure 5. *upd3* is induced in adult male and female flies upon exposure to 1% oxygen. Adult male and female flies were exposed to 2, 8 and 16 hours of 1% O_2 and gene expression of *upd3* and STAT target gene, *socs36e*, were measured by qRT-PCR. A, B) *Upd3* mRNA expression is upregulated by 8 hours of 1% O_2 exposure. C, D) Similarly, mRNA levels of *socs36e* are also induced. Data represent mean + SEM, N=4. Data points represent independent samples normalized to *bTub* mRNA levels.

3.1 Upd3/JAK/STAT Signaling is Induced in Hypoxia

Previous work in our lab has established a role for the Imd/Relish pathway in mediating hypoxia tolerance (Barretto et al., 2020). In this work, it was discovered that Imd/Relish, which canonically responds to gram-negative bacterial infection, was induced in hypoxia and the Toll

pathway, typically induced by gram-positive bacteria, was not. However, the role for the third branch of innate immunity – cytokine/JAK/STAT – had been relatively unexplored. Therefore, I wanted to investigate if the cytokine/JAK/STAT signaling pathway played a role in conferring a response to hypoxia in adult *Drosophila.* To do this, I subjected adult flies to 1% O_2 (hypoxia) or maintained at ambient oxygen (normoxia), then measured survival and collected samples for gene expression analysis (Figure 4). *Drosophila* larvae and adult females can survive up to ~24 hours of 1% O_2 with minimal effect on viability (Barretto et al., 2020). Therefore, exposed adults to 1% O_2 level because acute exposure to this level of oxygen results in adverse survival outcomes over, whereas higher concentrations such as 5% do not. This was useful because I could use survival as a functional readout for hypoxia tolerance.

To determine if the cytokine/JAK/STAT pathway was induced, I began by exposing adult male and female flies to 1% O_2 for 2, 8, and 16 hours and collected flies for gene expression analysis by qRT-PCR. These time points were chosen because they were sublethal for the w^{1118} flies. Firstly, I found *upd3* mRNA levels were induced in both male and female flies to comparable degrees (Figure 5A, B). Since this only indicated that transcripts of *upd3* were induced, I also measured STAT target gene *socs36e* as a functional readout of JAK/STAT pathway activation. When I did this, I also found upregulation of *socs36e* mRNA levels in males and females (Figure 5C, D) This induction occurs between 2 and 8 hours of hypoxia exposure compared to age-matched normoxia control animals.

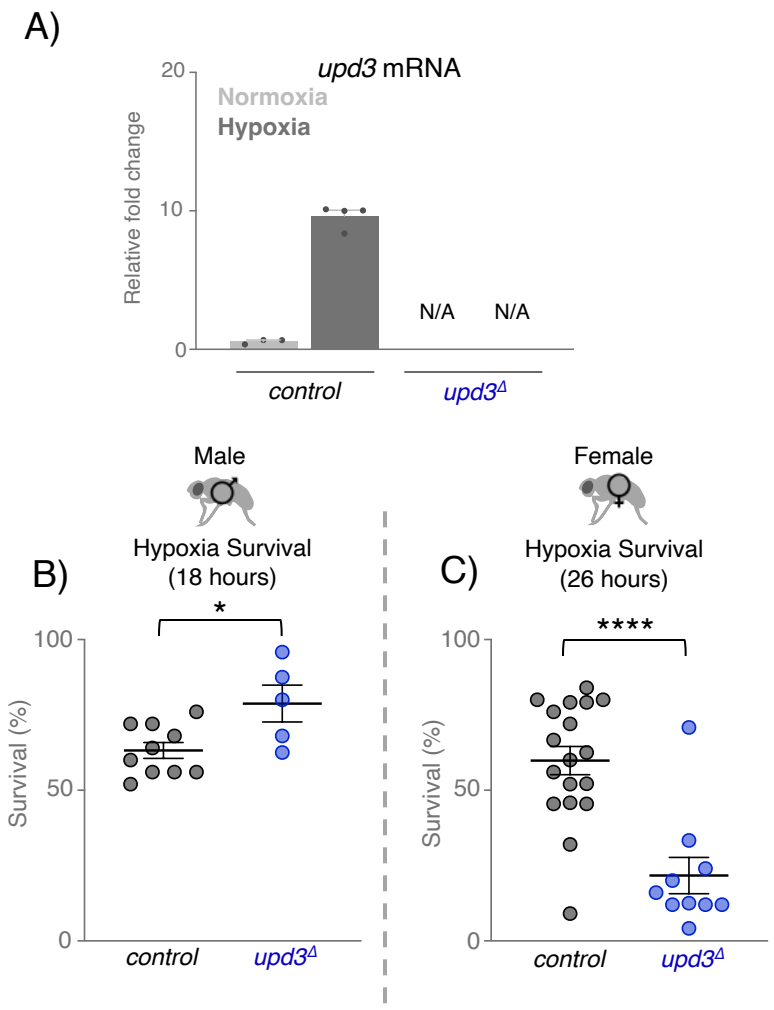

Figure 6. *upd3* is required for hypoxia tolerance in females but not males. A) Null *upd3*$^{\Delta}$ mutants have no detectable *upd3* expression in normoxia or 16 hours of 1% O_2. Data represents mean + SEM, N=4. Data points represent independent samples normalized to *bTub* mRNA levels. B, C) Hypoxia survival in *upd3*$^{\Delta}$ mutants is not negatively affected in males, but significantly reduces survival in females compared to controls (*w*^{1118}). Data represents mean ± SEM, N≥5. *p < 0.05, ****p < 0.00005, unpaired t-test. Data points represent independent samples.

3.1.1 Upd3 is Required for Hypoxia Tolerance in Adult Females, not Males

The role of the Upd3 ligand of the JAK/STAT pathway has been characterized in various stress contexts. These include nutrient stress, tumor stress, as well as infection and wounding stress (Agaisse et al., 2003; Pastor-Pareja et al., 2008; Woodcock et al., 2015). In each of these studies, whether *upd3* was helpful or harmful depended on the context. Therefore, I wanted to test the role for *upd3* in hypoxia tolerance. To do this, I used a viable $upd3^{\Delta}$ mutant line. This line contains an imprecise excision of the first three exons (including the ATG start codon) of the *upd3* gene. These null mutants were confirmed to have no *upd3* expression in normoxia or 16 hours of 1% O_2 (Figure 6A) and are ~100% viable in normoxia. To measure survival in hypoxia, I exposed age-matched 7-day old mated w^{1118} (control) and $upd3^{\Delta}$ adult male and female flies to 18 and 26 hours of hypoxia respectively. As previously stated, different exposure duration times were used because males were inherently less tolerant to hypoxia than females (See methods, pg. 32-33). Therefore, time of exposure was determined based on how long it took for the survival of control flies to reach 50-80%. Over the duration of hypoxia exposure, animals became anaesthetized. Therefore, survival was counted ~48 hours post hypoxia exposure to allow adequate time to recover. $upd3^{\Delta}$ mutant males did not show a decrease in their survival in hypoxia compared to w^{1118} control males (Figure 6B), however female $upd3^{\Delta}$ mutants showed significant reduced survival compared to their w^{1118} control counterparts (Figure 6C). These results suggest a sexually dimorphic requirement for *upd3* in mediating hypoxia tolerance whereby *upd3* is required for female, but not male survival. Given this requirement for *upd3* in females, I was interested in mechanisms involved in mediating this tolerance. As such, I focused my remaining experiments on asking what *upd3* was doing and how it might be regulated in female flies.

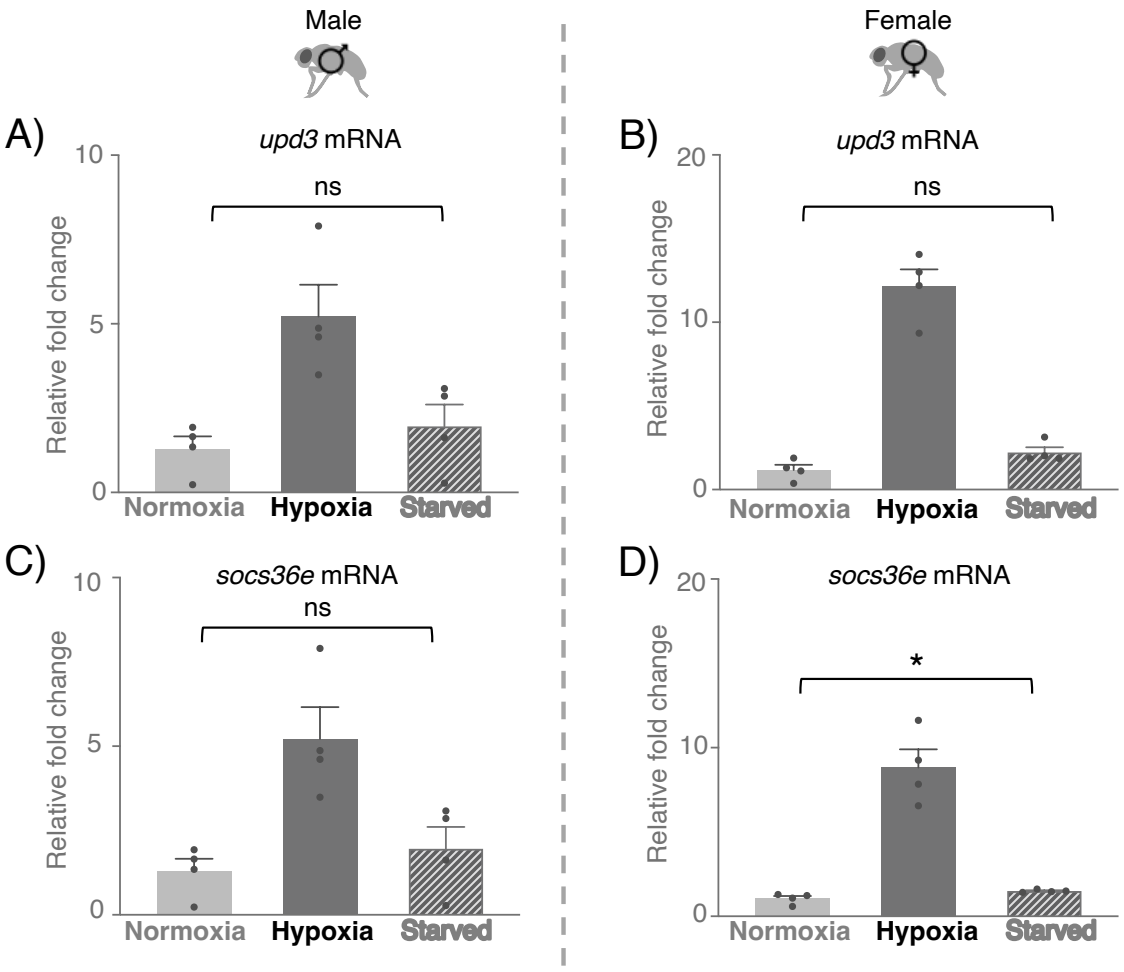

Figure 7. Starvation does not induce *upd3* or STAT target genes. Adult male and female flies were exposed to normoxia, 16 hours of 1% O_2 or 16 hours starvation. Gene expression of *upd3* and *socs36e* were measured by qRT-PCR. A, B) Upon starvation, there is no significant change in *upd3,* C, D) or *socs36e* gene expression. Data represents mean + SEM, N=4. ns not significant, *$p < 0.05$, unpaired t-test. Data points represent independent samples normalized to *bTub* mRNA levels.

3.1.2 Hypoxia-induced Cessation of Feeding Does Not Contribute to Upd3 Induction

Others and I had observed that flies exposed to 1% O_2 rapidly immobilize, enter a suspended animation-like state, and cease all feeding for the duration of hypoxia exposure (Habib et al.,

2021). To rule out the possibility that starvation may be responsible for *upd3* induction I observe in hypoxia, I set up three experimental groups in parallel: control (fed, normoxia), hypoxia (fed, 1% O_2 for 16 hours), and starved (starved for 16 hours, normoxia). After treatment, I collected flies for qRT-PCR analysis. In both adult male and female flies, starvation for 16 hours did not induce *upd3* or *socs36e* mRNA levels when compared to controls (Figure 7A-D). This suggests that the activation of JAK/STAT signaling upon exposure to 1% O_2 is due to the inadequate oxygen supply, not the cessation of feeding which concomitantly occurs.

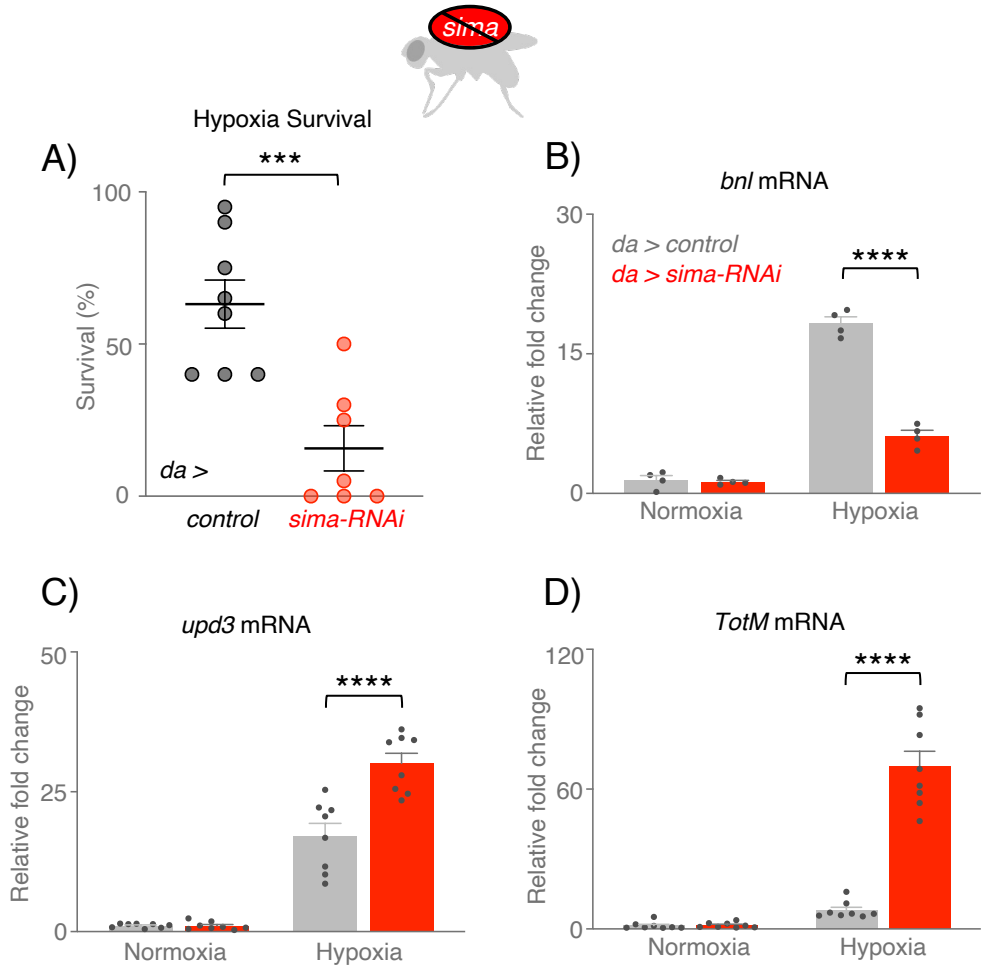

Figure 8. Sima/HIF-1α is required for hypoxia tolerance and restrains excess *upd3* signaling. Ubiquitous Sima knockdown (*da>sima-RNAi)* and control (*da>control)* adult females were exposed to 1% O_2. Survival and gene expression were then measured. A) Whole-body Sima knockdown (*da>sima-RNAi)* animals had a significant reduction in overall hypoxia survival compared to controls (*da>control)*. Data represents mean ± SEM, N=7-8. ***p < 0.0005, unpaired *t*-test. Data points represent individual samples. B) Induction of *Drosophila* FGF-like factor *bnl,* a Sima target gene, is blocked in Sima knockdown (*da>sima-RNAi)* flies compared to controls (*da>control)* in hypoxia. Data represents mean + SEM, N=4. ****p < 0.00005, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *bTub* mRNA levels. C, D) *Sima* is not required for the induction of *upd3* seen in hypoxia, but rather *upd3* levels are further induced when *sima* is knocked down ubiquitously (*da>sima-RNAi*) compared to controls (*da>control*). Data represents mean + SEM, N=8. ***p < 0.0005, ****p < 0.00005, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *bTub* mRNA levels.

3.2 Sima/HIF-1α Knockdown Decreases Hypoxia Tolerance

In both murine and fly models, HIF-1α has been shown to be required for hypoxia tolerance (Semenza, 2012). This requirement is largely due to the role HIF-1α plays in regulating hypoxia responsive gene expression, allowing various adaptations in low oxygen. Given this established requirement for Sima/HIF-1α in low oxygen, I wanted to confirm this in my own hands by using ubiquitous Sima knockdown flies. I exposed ubiquitous Sima knockdown (*da>sima-RNAi)* and control (*da>control)* female flies to hypoxia and compared their survival. Upon doing so, I found that *da>sima-RNAi* flies had significantly reduced survival compared to *da>control* flies in hypoxia (Figure 8A). In normoxia, both genotypes had ~99% viability. As a proxy to confirm Sima knockdown, I measured Sima target gene; the FGF-like factor *branchless (bnl).* When I did

this, I found that ubiquitous Sima knockdown flies (*da>sima-RNAi)* had reduced induction of *bnl* mRNA levels compared to controls (*da>control)* in hypoxia (Figure 8B). Together, these results confirm the importance of Sima expression for adult hypoxia survival and efficacy of the UAS Sima RNAi line to provide a strong knockdown of Sima function.

3.3 Sima/HIF-1α Restrains Excess Upd3 Induction to Promote Hypoxia Survival

One of the best characterized transcriptional regulators in hypoxia is activation of HIF-1 (Semenza, 2011). Therefore, I wanted to investigate whether HIF-1α was the transcription factor required for *upd3* induction in hypoxia. To test this, I used the ubiquitous *da-Gal4* driver to express a *UAS sima-RNAi* line in the whole animal (*da>sima-RNAi)* and compare *upd3* levels in hypoxia to controls (*da>control)*. In Sima knockdown adults (*da>sima-RNAi*), *upd3* (Figure 8A) and *TotM* mRNA levels (Figure 8B) were induced to an even greater degree compared to controls *(da>control)* in hypoxia. Interestingly, this suggests that Sima is not required for *upd3* induction in hypoxia, but instead functions to negatively regulate Upd3 levels.

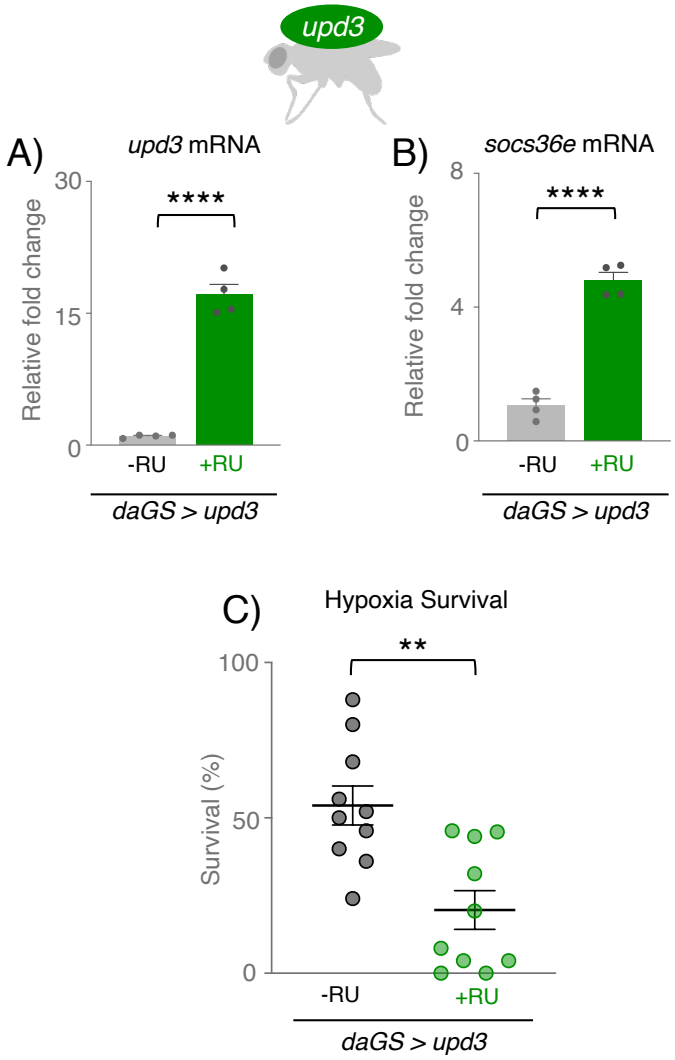

Figure 9. *upd3* overexpression leads to reduced hypoxia tolerance. Control (*daGS>upd3*, fed control food, -RU) and ubiquitous *upd3* overexpression (*daGS>upd3*, fed RU486 food, +RU) were exposed to hypoxia and gene expression of *upd3* and *socs36e* as well as hypoxia survival were measured. A, B) Ubiquitous *upd3* overexpression leads to significant increase in both *upd3* and *socs36e* mRNA levels compared to controls. Data represents mean + SEM, N=4. ****$p < 0.00005$, unpaired *t*-test. Data points represent independent samples normalized to *RpS24* mRNA levels. C) Genetic overexpression of *upd3*, mimicking the phenotype observed in ubiquitous *sima*-RNAi animals, leads to reduced survival in hypoxia. Data represents mean ± SEM, N=10. **$p < 0.005$, unpaired *t*-test. Data points represent individual samples.

The immune system plays a crucial role in fighting environmental insults like pathogens, wounds, and toxins. However, once the insult has been cleared or neutralized, it is equally as crucial for the immune system to return to a non-induced state. Dysfunction in this return to normal is characteristic of immune disorders and has complications beyond the initial immune challenge. Therefore, I hypothesized that one role of Sima in hypoxia is to limit too much Upd3 signaling to promote survival.

To test if excess Upd3 was deleterious to the flies, I genetically overexpressed *upd3* ubiquitously (*daGS>upd3)* in adult female flies using the *GeneSwitch-Gal4* system, which induces the transgene *UAS-upd3* only when flies are fed mifepristone (RU486). This system allows for induction after the developmental stage to avoid possible developmental effects (Osterwalder et al., 2001). I confirmed by qRT-PCT that the gene expression system was in fact upregulating *upd3* (Figure 9A) and *socs36e* (Figure 9B) mRNA levels. I then exposed *upd3* overexpression (*daGS>upd3,* fed RU486*)* and control flies (*daGS>upd3*, fed vehicle control*)* to hypoxia to measure survival. I found that *upd3* overexpression led to decreased survival in hypoxia (Figure 9C). Together with the result from Figure 6C, these results suggest that *upd3* signaling is both required for mediating hypoxia tolerance in female adult flies, but also too much cytokine signaling is detrimental. This notion is consistent with the literature on immune signaling which has emphasized the need for physiological mechanisms to both induce and restrain cytokine signaling to fine-tune immune responses.

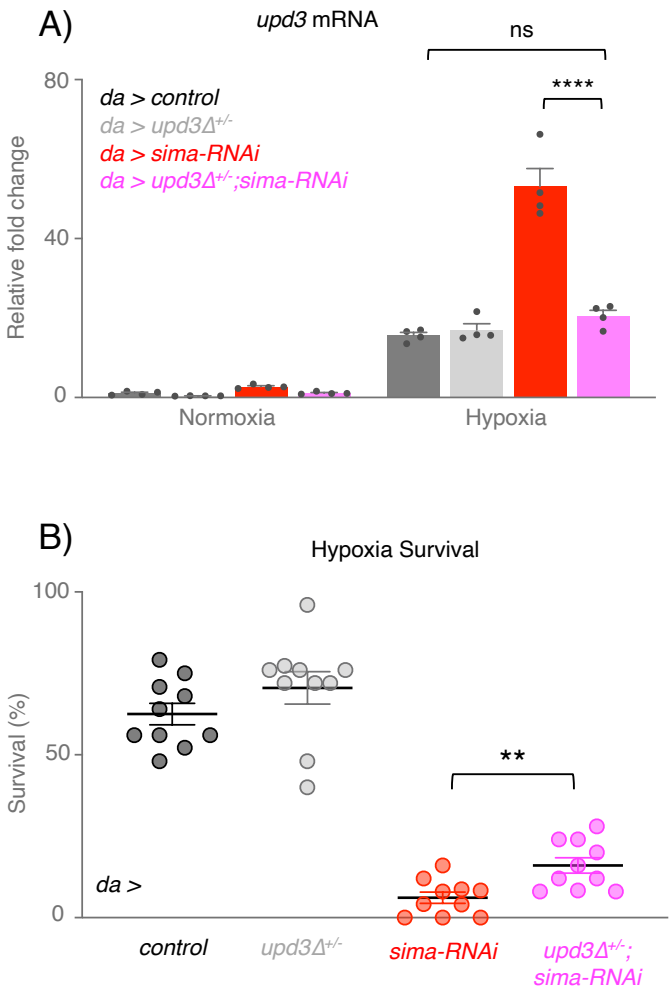

Figure 10. Reducing *upd3* expression partially rescues lethality in Sima knockdown animals. Control *(da>control)*, upd3Δ heterozygote (*da>upd3Δ*$^{+/-}$), *sima* knockdown (*da>sima-RNAi*) and *upd3* heterozygote *sima* knockdown (*da>upd3Δ*$^{+/-}$*;sima-RNAi*) adult females were put in hypoxia (1% O_2) or normoxia. Gene expression and survival were measured after 16 and 24 hours respectively. A) Hyper-induction of *upd3* mRNA levels seen in *sima* knockdown animals is abrogated in an upd3Δ$^{+/-}$ background. Data represents mean + SEM, N=4. ns not significant, ****$p < 0.00005$, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *RpS24* mRNA levels. B) Reduction of *upd3* induction partially rescues lethality in *sima* knockdown animals. Data represents mean ± SEM, N≥8. **$p < 0.005$, unpaired *t*-test. Data points represent individual samples.

Given the previous results, I wondered whether the lethality seen in ubiquitous Sima knockdown animals may be caused by too much Upd3 cytokine signaling. To directly test whether excess *upd3* was responsible for lethality of Sima knockdown animals, I looked to see if loss of one copy of *upd3* might affect survival of *da>sima-RNAi* flies. First, I confirmed by qRT-PCR that *upd3* mRNA levels were reduced in these animals. As shown previously, Sima knockdown animals (*da>sima-RNAi)* had super-induced levels of *upd3* mRNA in hypoxia compared to control (*da>control)* (Figure 10A). Upd3 heterozygotes with Sima knockdown (*da>upd3*$\Delta^{+/-}$*;sima-RNAi*) showed a reversal of this elevated *upd3* induction, with *upd3* mRNA levels returning to the level of controls (*da>control)* in hypoxia (Figure 10A).

I then examined hypoxia survival in these animals. As demonstrated previously in Figure 8C, ubiquitous Sima knockdown (*da>sima-RNAi)* animals had reduced survival in hypoxia compared to controls (*da>control)* (Figure 10B). However, Upd3 heterozygote Sima knockdown animals (*da>upd3*$\Delta^{+/-}$*;sima-RNAi*) showed increased survival compared to the Sima knockdown animals (*da>sima-RNAi)* in hypoxia (Figure 10B). HIF-1α/Sima is a main regulator of transcriptional response to hypoxia, coordinating metabolic, vascular, and proliferative adaptations (Semenza, 2011). The results described above suggest that part of the lethality seen in Sima knockdown animals is caused by exaggerated *upd3* induction in hypoxia.

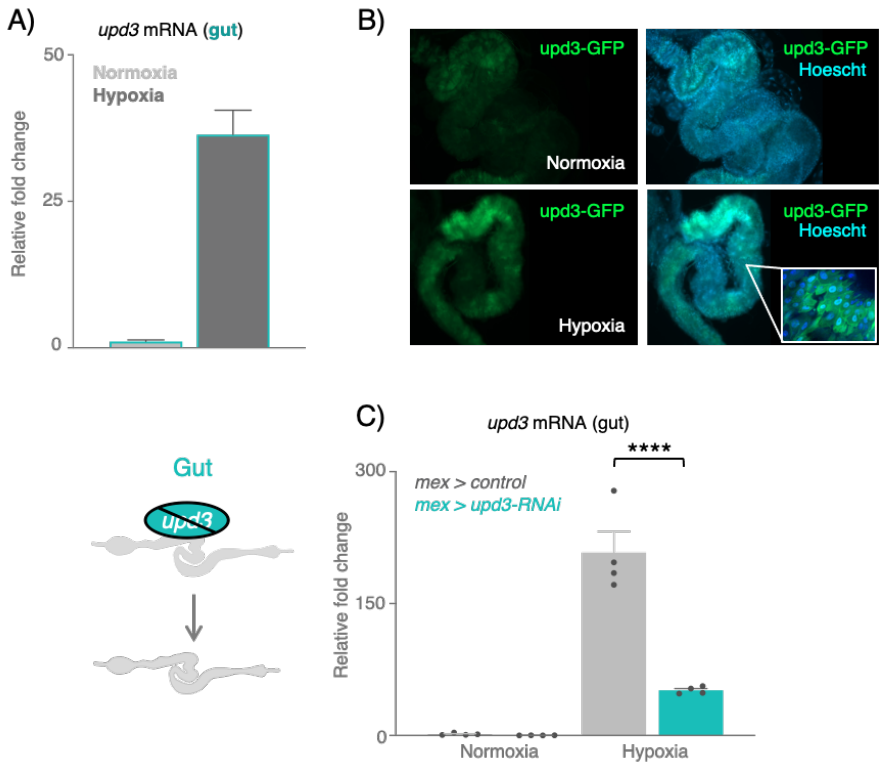

Figure 11. *upd3* is induced in female guts upon exposure to hypoxia. A) Adult females (*w*^{1118}) maintained in normoxia or exposed to 16 hours of 1% O_2 were dissected for qRT-PCR analysis. Guts showed strong induction of *upd3* mRNA levels in hypoxia. Data represents mean + SEM, N=4. Normalized to *eIF-2α* mRNA levels. B) Female flies from an upd3-GFP reporter line were maintained in normoxia or exposed to 1% O_2 for 16 hours. Tissues were dissected for visualization of GFP expression. Representative images of whole guts expressing *upd3*-GFP (*upd3Gal4, UAS-GFP/Cyo*) with Hoescht-stained nuclei. GFP expression in the enterocytes of the gut is increased upon exposure to hypoxia. C) *Upd3* was knocked down using an enterocyte-specific driver, then adult females were maintained in normoxia or exposed to 1% O_2 for 16 hours and guts immediately dissected for qRT-PCR analysis. Enterocyte Upd3 knockdown (*mex>upd3-RNAi)* led to ~80% blocking of *upd3* mRNA induction compared to controls (*mex>control)* in hypoxia. Data represents mean + SEM, N=4. ****$p < 0.00005$, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *RpS24* mRNA levels.

3.4 Upd3 is Strongly Induced in the Gut in Hypoxia

All results described thus far has been with whole-animal manipulations of Upd3. Studies of *upd3* responses in stresses such as infection and wounding, tumor stress, and nutrient stress (Agaisse et al., 2003; Pastor-Pareja et al., 2008; Woodcock et al., 2015) show that although the requirement for *upd3* in mediating a positive or negative effect on organismal tolerance to these stresses can differ, a common theme through each is the fact that Upd3 acts as a long-range signaling molecule which facilitates tissue-to-tissue communication. Therefore, I wanted to investigate what tissue(s) are producing *upd3* in hypoxia.

One well-studied organ that produces Upd3 is the fly intestine. I therefore investigated a potential role for gut derived Upd3 in hypoxia. To do this, I exposed w^{1118} females to normoxia or 16 hours of 1% hypoxia and dissected guts for qRT-PCR analysis. When I did this, I found that the gut showed strong upregulation of *upd3* mRNA levels in hypoxia (Figure 11A). Next, I used flies carrying an *upd3* GFP transcriptional reporter line (*upd3Gal4, UAS-GFP*). I exposed these flies to either normoxia or hypoxia to visualize GFP expression in the intestine. Female flies exposed to hypoxia had stronger GFP expression in their guts compared to normoxia controls (Figure 11B). Higher resolution confocal microscopy revealed that it was specifically the large epithelial enterocyte cells of the gut which were strongly expressing the GFP and were the main *upd3*-producing cell type. To confirm this using a genetic approach, I knocked down Upd3 using an enterocyte-specific driver (*mex>upd3-RNAi)* and dissected whole intestines to measure *upd3* mRNA levels. I found that enterocyte Upd3 knockdown (*mex>upd3-RNAi)* resulted in ~80% reduction in *upd3* mRNA induction compared to controls (*mex>control)* in

hypoxia (Figure 11C). Together, these results suggest that gut enterocytes strongly induce *upd3* mRNA levels in hypoxia.

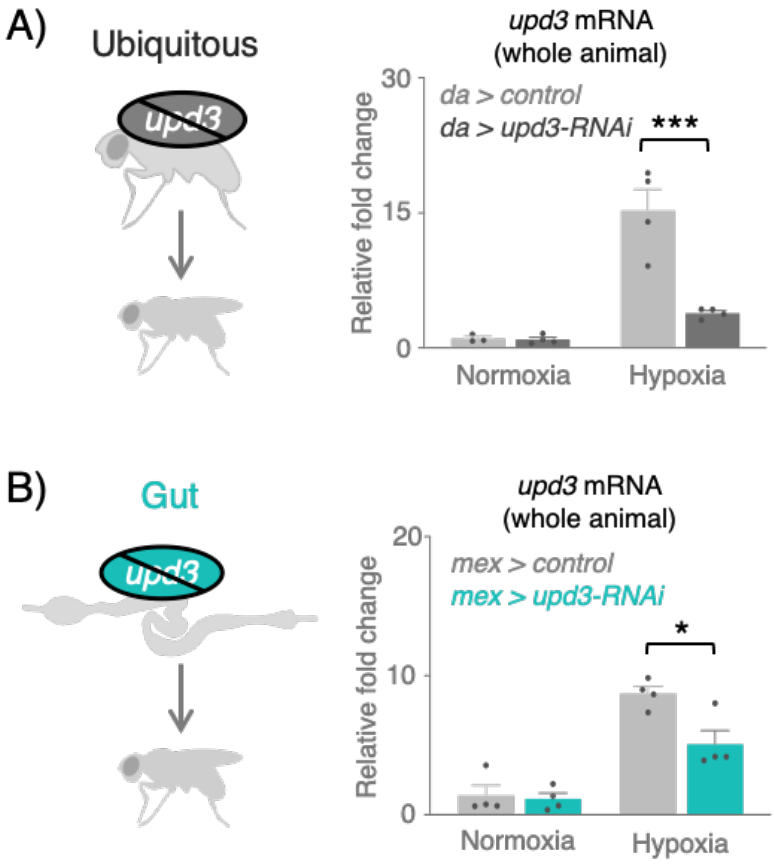

Figure 12. Genetic knockdown of *upd3* in gut and leads to significant blunting of *upd3* induction in hypoxia. A) Ubiquitous knockdown of *upd3* (*da>upd3-RNAi*) abrogates *upd3* induction seen in hypoxia compared to controls (*da>control*). B) Gut-specific knockdown of *upd3* (*mex>upd3-RNAi*) also significantly blocks whole organism *upd3* mRNA levels in hypoxia when compared to controls (*mex>control*). Data represents mean + SEM, N=4. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *bTub* mRNA levels.

Next, I was interested in looking at contribution of gut Upd3 to the whole animal increase in Upd3 levels observed in hypoxia. Initially, I knocked down Upd3 ubiquitously (*da>upd3-RNAi)* and measured *upd3* mRNA levels in hypoxia. I found that these animals showed a ~80% reduction in their *upd3* induction in hypoxia compared to controls (*da>control)*, confirming that the RNAi line was effective in achieving Upd3 knockdown (Figure 12A). I next wanted to test how much Upd3 from the gut was contributing to the induction seen in hypoxia. To do this, I crossed the enterocyte-specific *mex-Gal4* line to the same transgenic *UAS upd3-RNAi* line previously used. The progeny from this cross expressed the RNAi to Upd3 specifically in the gut. I then measured whole animal *upd3* mRNA levels in hypoxia. I observed that gut Upd3 knockdown (*mex>upd3-RNAi)* lead to a ~50% reduction in *upd3* mRNA levels in hypoxia compared to controls (*mex>control).* Together, these results suggest that gut-derived *upd3* is a significant contributing source of total *upd3* in hypoxia.

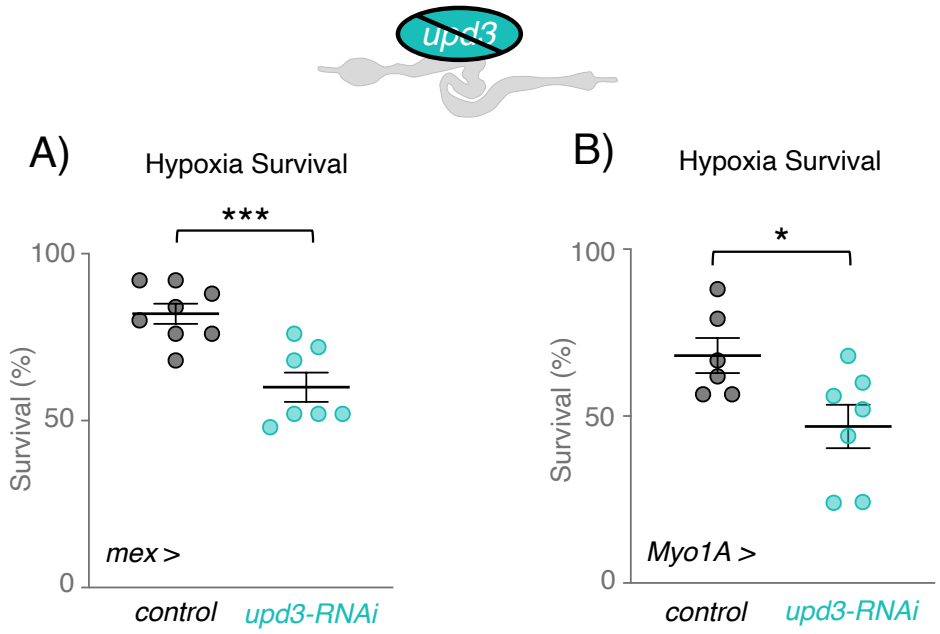

Figure 13. Gut-derived *upd3* is required for female hypoxia survival. A, B) *Upd3* knockdown in the gut using two enterocyte tissue drivers (*mex>upd3-RNAi, Myo1A>upd3-RNAi*) leads to significant reduction in hypoxia survival (26 hours) compared to respective control genotypes (*mex>control, Myo1A>control*). Data represents mean ± SEM, N≥6. *$p < 0.05$, ***$p < 0.0005$, unpaired *t*-test. Data points represent individual samples.

3.4.1 Gut Upd3 is Required for Hypoxia Tolerance

After demonstrating that the gut was a main source of *upd3* in hypoxia, I next wanted to investigate its functional role. To do this, I first took the same gut Upd3 knockdown (*mex>upd3-RNAi)* and control (*mex>control)* flies, exposed them to hypoxia and measured survival. In normoxia, these flies were ~99% viable. Upon exposure to hypoxia, I found that the gut Upd3 knockdown flies (*mex>upd3-RNAi)* had reduced survival compared to controls (*mex>control)* (Figure 13A). To further confirm this result, I used *Myo1A-Gal4,* another gut enterocyte-specific driver, to knockdown Upd3 and test survival. Like the previous result*, Myo1A>upd3-RNAi*

animals showed reduced survival compared to controls (*Myo1A>control)* (Figure 13B) and were ~99% viable in normoxia. Taken together with the results in Figure 12, these results demonstrate that the gut is a major source of *upd3* in hypoxia which is required to mediate tolerance.

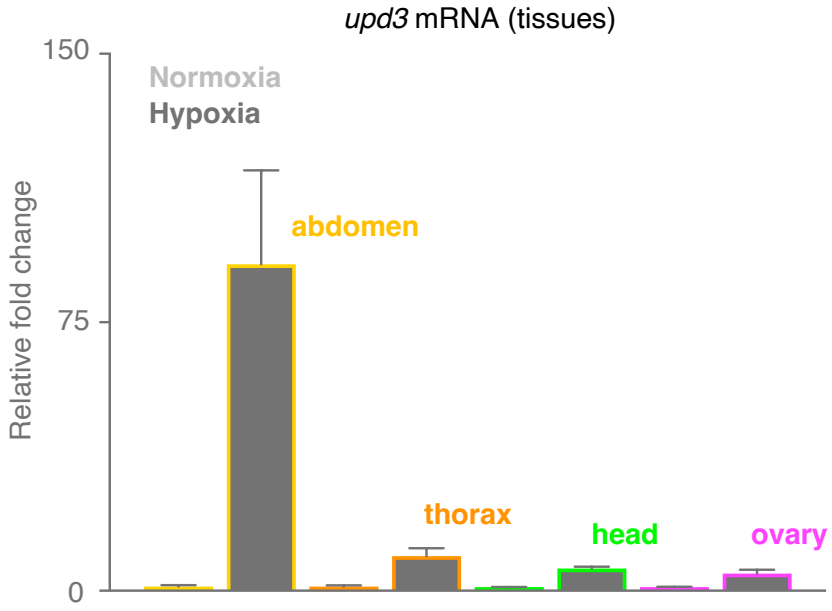

Figure 14. *upd3* induction in hypoxia is also seen in the abdomen and other adult female tissues. Remaining tissue types (abdomen, thorax, head, ovary) from w^{1118} adult females were dissected and isolated for qRT-PCR analysis to measure *upd3* induction upon exposure to 16 hours of 1% O_2. All tissues showed induction of *upd3* mRNA in hypoxia but was strongest in the abdomen. Data represents mean + SEM, N=4. Normalized to *eIF2α* mRNA levels.

3.5 Upd3 is Also Induced in the Fat Body-Containing Abdomen and Other Tissues

While the previous results provide strong support for the idea that gut-derived *upd3* is the main mediator of hypoxia tolerance, I also wanted to investigate *upd3* expression in other tissues. I

dissected tissues from adult female w^{1118} flies in hypoxia and compared *upd3* mRNA levels to controls maintained in normoxia. The tissues I dissected were the abdomen (enriched for fat body), thorax (enriched for muscle), head (enriched for brain), and the ovaries. The abdomen samples showed a strong induction of *upd3* mRNA levels in hypoxia, with the other tissues showing moderate induction (Figure 14). This suggests that the abdomen is also a major source of *upd3* in hypoxia.

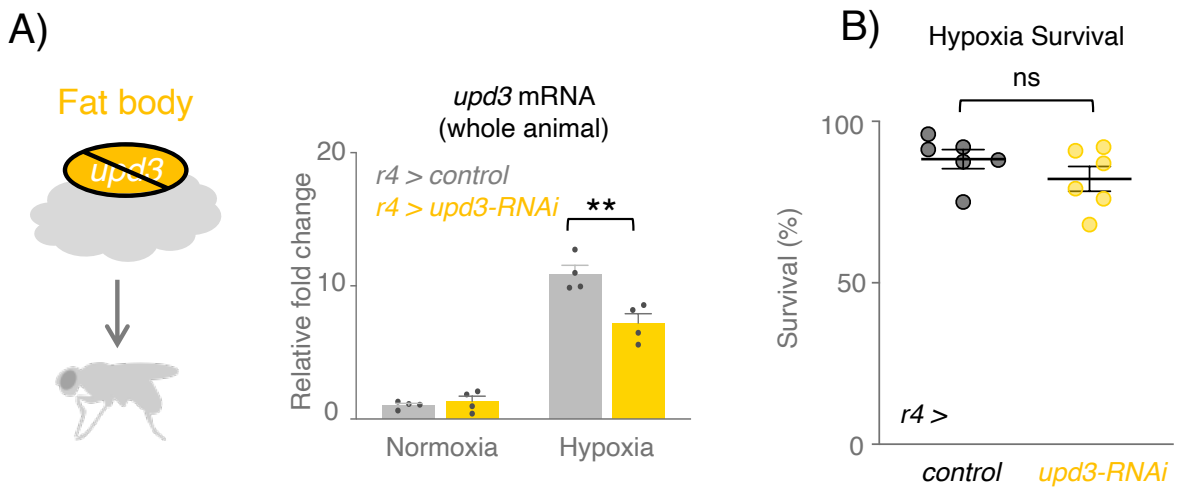

Figure 15. *Upd3* in the female fat body is not required for hypoxia tolerance. Fat body Upd3 knockdown (*r4>upd3-RNAi)* and control (*r4>control)* adult female flies were placed in 1% O_2, then collected for gene expression analysis or survival measured. A) *Upd3* knockdown specifically fat body (*r4>upd3-RNAi*) significantly reduces *upd3* induction in hypoxia. Data represents mean + SEM, N=4. **p < 0.005, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *act5C* mRNA levels. B) After 26 hours of hypoxia exposure, *upd3* knockdown in the fat body (*r4>upd3-RNAi*) does not significantly affect survival compared to controls (*r4>control*). Data represents mean ± SEM, N=6. *ns* not significant, unpaired *t*-test. Data points represent individual samples.

I therefore performed genetic knockdown of fat body Upd3 to test its role in hypoxia tolerance. Fat body knockdown of Upd3 (*r4>upd3-RNAi)* lead to ~30% reduction in *upd3* mRNA levels compared to controls (*r4>control)* in hypoxia (Figure 15A). To test if fat body Upd3 was required for hypoxia tolerance, I took fat body Upd3 knockdown animals (*r4>upd3-RNAi)* and controls (*r4>control)*, exposed them to hypoxia and then measured survival. When I did this, I observed no significant difference in their survival (Figure 15B). Together with previous results, this suggests that while other tissues such as the fat body may be responsible for high induction of *upd3* in hypoxia, it is specifically gut-derived *upd3* which mediates hypoxia tolerance.

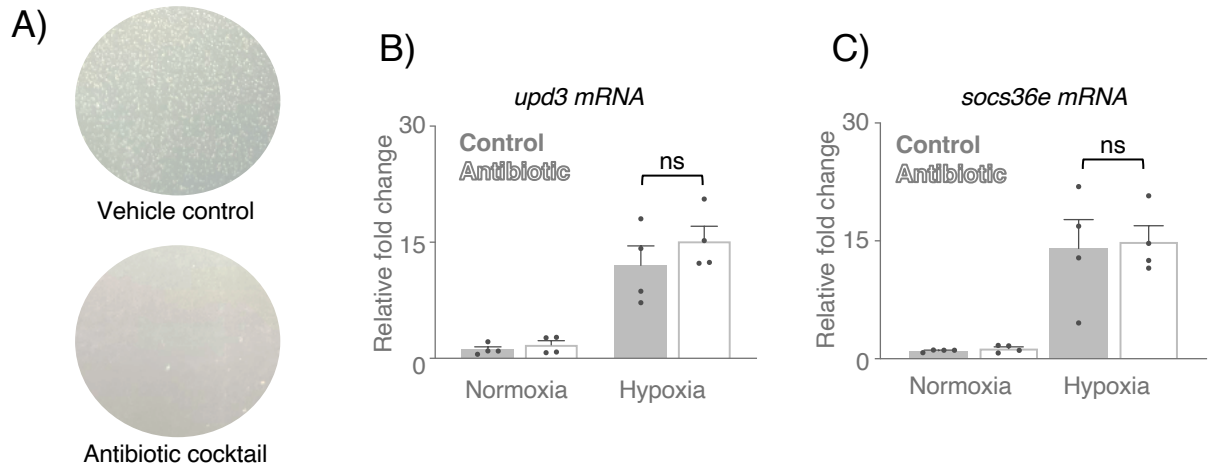

Figure 16. The gut microbiome is not required for *upd3* induction in hypoxia. Adult females were fed food supplemented with antibiotics or vehicle control for 5 days. Next, flies were exposed to 1% O_2 for 16 hours or maintained in normoxia, then collected for qRT-PCR analysis. Fly lysates were streaked on LB plates that were kept at 37°C for 1-2 days to observe bacterial colony growth. A) Fewer colonies formed from lysate derived from flies fed the antibiotic cocktail compared to vehicle control-fed flies. B, C) Adult axenic flies showed no significant difference in *upd3* and *socs36e* mRNA levels compared to controls when exposed to 1% O_2. Data represents mean + SEM,

N=4. ns not significant, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *RpS24* mRNA levels.

3.6 The Gut Microbiome is Not Required for Upd3 Induction in Hypoxia

In response to other stress, like a high-fat diet (HFD), it had been shown that the *Drosophila* gut microbiota become dysbiotic, induce inflammation, and disrupt Upd3/JAK/STAT signaling (Meng et al., 2023). The intestinal stem cell response to inflammation caused by HFD has also been shown to be microbiome-dependent, as flies lacking a microbiome no longer elicited the response (von Frieling et al., 2020). Therefore, I wanted to study whether an intact microbiome contributes to *upd3* induction in hypoxia. Since I was working with adult flies as my model, I raised larvae under normal conditions and then generated germ free adults. I used w^{1118} adult female flies and maintained them on food containing either a cocktail of antibiotics or a vehicle control. To confirm that I had successfully generated adult flies which were germ-free, I streaked the fly lysates on LB plates and monitored bacterial colony growth. I was able to observe that the antibiotic cocktail treated fly lysates had distinctly less bacterial colonies compared to vehicle control treated fly lysates (Figure 16A). After confirming the efficacy of the antibiotic treatment, I exposed flies from both groups to either hypoxia or normoxia to collect for gene expression analysis. I found that germ-free flies did not significantly differ from control flies in their induction of either *upd3* (Figure 16B) or *socs36e* mRNA levels (Figure 16C) in hypoxia. This suggests that the gut microbiome is not required for activation of the Upd3/JAK/STAT pathway in hypoxia.

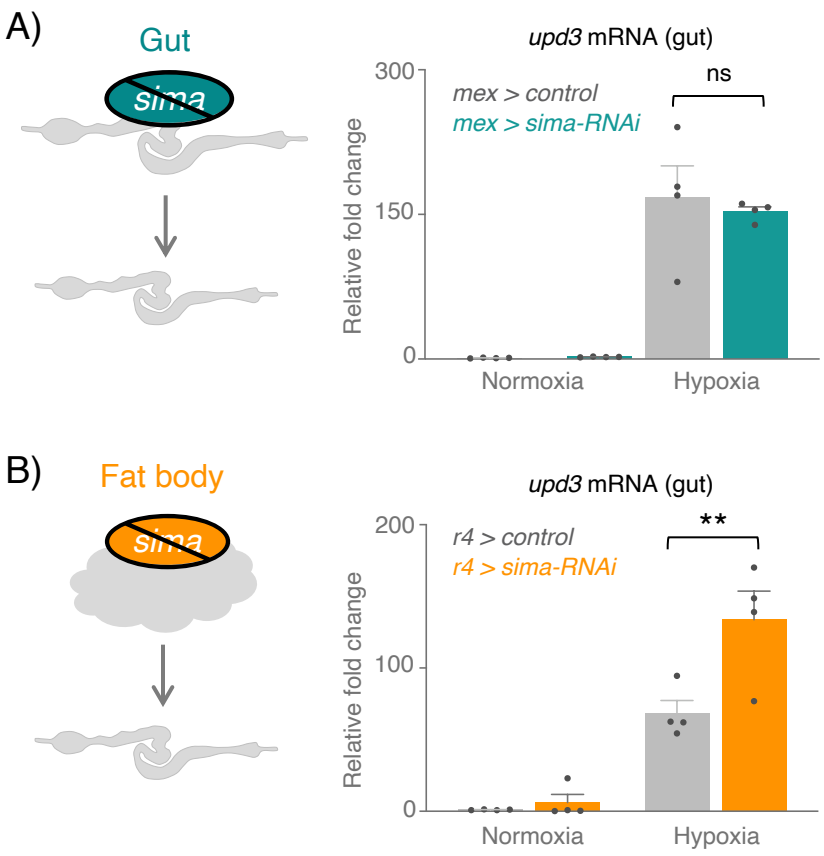

Figure 17. HIF-1α/Sima in the fat body restrains Upd3 signaling from the gut. Sima was knocked down in the gut and fat body, then adult females were placed in 1% O_2 for 16 hours and guts were immediately dissected for qRT-PCR analysis. A) Gut Sima knockdown *(mex>sima-RNAi)* in adult female guts showed no significant difference in *upd3* mRNA levels in hypoxia compared to control *(mex>control).* B) Fat body Sima knockdown *(r4>sima-RNAi)* leads to significant blocking of *upd3* mRNA levels in the gut compared to control *(r4>control).* Data represents mean + SEM, N=4. **$p < 0.005$, ns not significant, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *5S* mRNA levels.

3.7 Fat Body Sima Negatively Regulates Gut Upd3 Levels

Next, I was interested in investigating what signals regulate gut Upd3. I decided to approach this by knocking down candidate genes. Since I had shown that Sima functions to block induction of Upd3 in hypoxia, I wanted to investigate in which tissue Sima was acting. After establishing the gut as the main source of Upd3 required for mediating hypoxia tolerance, I hypothesized that Sima in the gut was antagonizing gut Upd3. To test this, I used the gut driver *mex-Gal4* and crossed it to a *UAS sima-RNAi* line to generate gut-specific Sima knockdown. I then isolated the guts from adult females in normoxia and hypoxia to measure *upd3* mRNA levels. When I did this, I saw that *upd3* mRNA levels in gut Sima knockdown flies (*mex>sima-RNAi*) did not significantly differ compared to control flies (*mex>control*) in hypoxia (Figure 17A). This suggests that gut Sima does not control gut Upd3.

In larvae, the fat body has been shown to sense hypoxia directly through Sima and release factors which slow growth (Texada et al., 2019). The *Drosophila* fat body also acts as a central integration center for signals to and from other tissues. Based on this, I examined a potential role for fat body Sima. To do this, I generated fat body Sima knockdown flies (*r4>sima-RNAi*), exposed them to hypoxia and measured gut *upd3* mRNA levels. I found that fat body Sima knockdown (*r4>sima-RNAi*) guts showed a significant increase in Upd3 induction in hypoxia compared to control (*r4>control*) guts (Figure 17B). Together, these results suggest that Sima in the fat body acts as a hypoxia sensor to control Upd3 in the gut. It also suggests that there is a Sima-dependent signaling molecule which is communicating between tissues. Further research

would need to be conducted to determine what this signal may be and how it controls gut *upd3* levels.

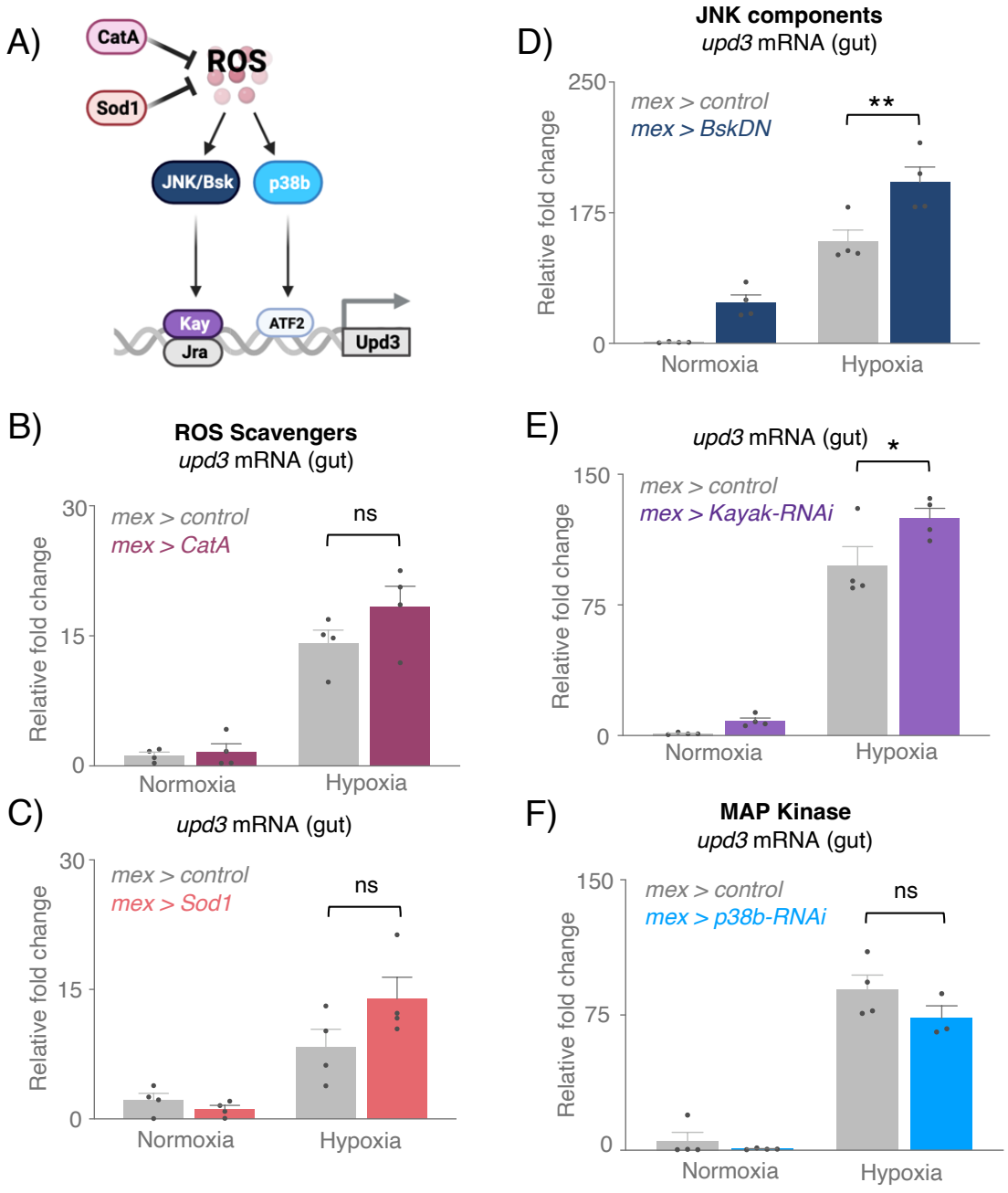

Figure 18. Hypoxia induction of gut *upd3* is independent of ROS/JNK/p38 signaling. A) ROS/JNK/p38 signaling pathway in *Drosophila.* B, C) Overexpression of ROS scavengers (*mex>Sod1, mex>CatA*) and D, E) JNK components (*mex>BskDN, mex>Kayak-RNAi*) are also unable to block in induction of *upd3* in the gut upon exposure to 1% O_2 compared to controls (*mex>control*). F) Knockdown of gut MAP Kinase p38b (*mex>p38b-RNAi*) does not affect *upd3* induction in hypoxia compared to controls (*mex>control).* Data represents mean + SEM, N=4. *$p < 0.05$, **$p < 0.005$, ns not significant, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *RpS24* mRNA levels.

After ruling out Sima as the transcription factor responsible for Upd3 induction in hypoxia, I decided to look at other candidate signaling pathways that might mediate the hypoxia induction of gut Upd3. In the gut, Upd3 has been shown to be regulated by pathways including MAPK, ROS, JNK, BMP, and Hippo (Houtz et al., 2017; Chakrabarti & Visweswariah, 2020; Jiang et al., 2009; H. Li et al., 2013; Staley & Irvine, 2010). Using the versatility of *Drosophila* genetics, I investigated the role of each of these pathways on Upd3 induction in hypoxia. I did so by using the gut-specific driver *mex-Gal4* crossed to various UAS lines to manipulate the pathways of interest. I then dissected guts from the adults exposed to hypoxia or normoxia and compared *upd3* mRNA levels to their appropriate controls. I hypothesized that one or more of these pathways might be responsible for the gut *upd3* induction in hypoxia.

3.8 Induction of Gut Upd3 is Independent of ROS/JNK/p38 Signaling

I decided to start by investigating the role of reactive oxygen species (ROS) in hypoxia *upd3* upregulation. In *Drosophila,* ROS can activate JNK/Bsk and MAP Kinase p38b, resulting

upregulation of *upd3* (Figure 18A). Antioxidant genes *catalase* and *superoxide dismutase* scavenge hydrogen peroxide and superoxide anions, respectively (Krumova & Cosa, 2016). Given this, I decided to genetically overexpress these two antioxidant genes in the gut to determine if ROS is the trigger that causes induction of *upd3* in hypoxia. Again, I used *mex-Gal4* to specifically overexpress Catalase A and Superoxide Dismutase 1 in the gut using *UAS-CatA* and *UAS-Sod1* respectively. When I exposed gut Catalase A overexpression (*mex>CatA)* animals to hypoxia and collected guts for qRT-PCR, I observed no difference in their *upd3* mRNA levels compared to controls *(mex>control)* (Figure 18B). Similar results were attained when I overexpressed Sod1 in the intestine (*mex>Sod1)* (Figure 18C).

To start look at downstream effectors of ROS, I used *mex-Gal4* to express a dominant negative version of JNK/Bsk (*mex>BskDN*) to effectively abrogate endogenous Bsk signaling. When I measured *upd3* mRNA levels of guts isolated from flies exposed to hypoxia or normoxia, I found that Bsk was not required for *upd3* induction but instead showed slight increase in *upd3* mRNA levels compared to controls (*mex>control)* (Figure 18D). I also performed gut knockdown of downstream target of JNK/Bsk, Kayak (*mex>kayak-RNAi)*, an AP-1 transcription factor complex component, to see if this alternative approach to knocking down JNK signaling would be sufficient to block induction of *upd3* in hypoxia. However, I found that this too failed to block *upd3* mRNA levels in hypoxia compared to controls (*mex>control)* (Figure 18E). Instead, gut Kayak knockdown lead to an even greater induction of *upd3,* like the result observed with *mex>BskDN* animals. Next, I tested a role for p38 kinase. I used *mex-Gal4* crossed to *UAS p38b-RNAi* to knockdown p38b specifically in the gut. I found that p38b knockdown in the gut (*mex>p38b-RNAi)* does not affect *upd3* induction in hypoxia when compared to control

(*mex>control)* levels (Figure 18F). Together these studies rule out a role for the ROS/JNK/p38 pathway in hypoxia mediated induction of gut *upd3*.

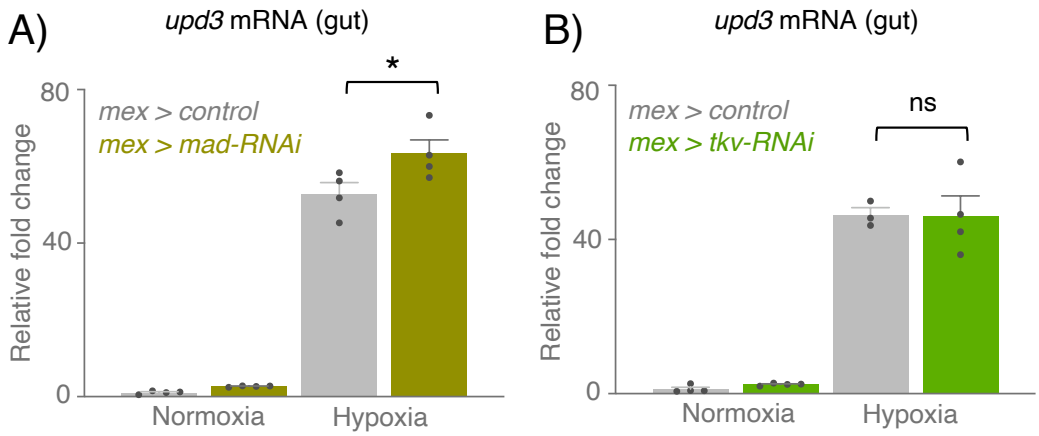

Figure 19. Hypoxia induction of gut *upd3* is independent of BMP/Mad signaling. Knockdown of *Drosophila* A) BMP-Smad (*mex>mad-RNAi*) and B) BMP Type I receptor (*mex>tkv-RNAi*) are unable to block in induction of *upd3* in the gut, when compared to controls (*mex>control*), upon exposure to 1% O_2. Data represents mean + SEM, N=3-4. ns not significant, *$p < 0.05$, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *RpS24* mRNA levels.

3.9 Induction of Gut Upd3 is Independent of BMP/Mad Signaling

Another candidate signaling pathway for gut *upd3* induction is the bone morphogen protein (BMP) branch of the TGF-β pathway. It has been previously shown that the BMP R-Smad, called Mad in flies, is necessary for *upd3* induction during infection (Houtz et al., 2017). I was therefore interested in studying its role in the context of hypoxia and *upd3* signaling. To look at this, I knocked down Mad in the intestine (*mex>mad-RNAi*) to measure gut *upd3* in hypoxia. I found that this did not block *upd3* signaling but instead slightly exacerbated *upd3* levels

compared to controls (*mex>control)* hypoxia (Figure 19A). I also tried knocking down BMP Type I receptor, Thickveins. Gut knockdown of Thickveins (*mex>tkv-RNAi)* did not affect *upd3* induction in hypoxia when compared to controls (*mex>control)* (Figure 19B). These results taken together suggest that the BMP signaling pathway is not required for gut *upd3* induction in hypoxia.

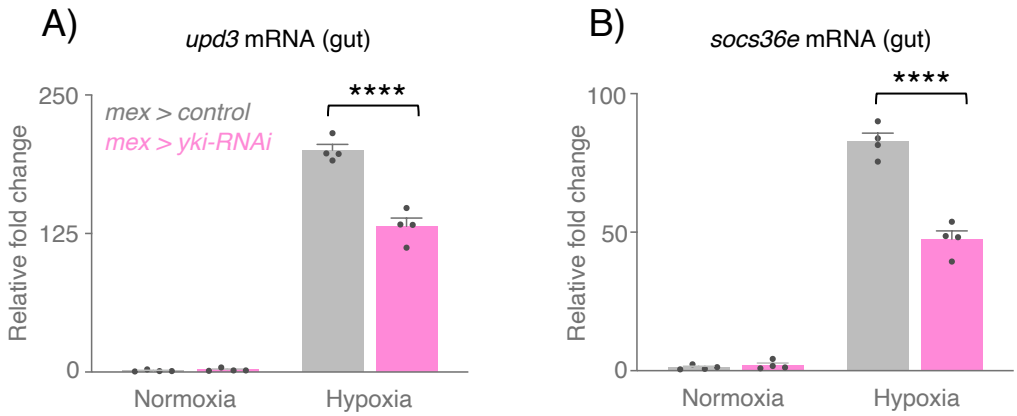

Figure 20. Gut-specific genetic manipulation of Yki partially blocks *upd3* induction in the gut. Control (*mex>control*) and gut-specific Yki knockdown (*mex>yki-RNAi)* adult females were placed in 1% O_2 for 16 hours and collected for gene expression analysis. A) Yki knockdown in the gut significantly blocks gut-derived *upd3* and B) *soc36e* mRNA levels in hypoxia. Data represents mean + SEM, N=4. ****p < 0.00005, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *eIF2α* mRNA levels.

3.10 Hippo/Yorkie Partially Responsible for Inducing Upd3 in Hypoxia

Finally, I tested another signaling pathway, the Hippo/Yorkie pathway, which has been previously implicated in *upd3* induction during infection (Houtz et al., 2017; Li et al., 2014). Inactivation of Hippo prevents phosphorylation of Yorkie, allowing it to enter the nucleus and

function as a co-transcription factor for target genes such as *upd3* (Oh & Irvine, 2010). As described previously, I used *mex-Gal4* to express *UAS yki-RNAi* and measured gene expression in hypoxia. In doing so, I observed that gut Yorkie knockdown (*mex>yki-RNAi)* flies had ~30% knockdown of *upd3* mRNA levels in hypoxia compared to control (*mex>control)* (Figure 20A). I also measured *socs36e* mRNA levels and saw that they too were reduced by ~30% (Figure 20B), suggesting that gut *upd3* under the control of Yorkie acts in an autocrine manner back on the gut. Furthermore, these results support the idea that Hippo/Yorkie is partially responsible for gut *upd3* induction in hypoxia.

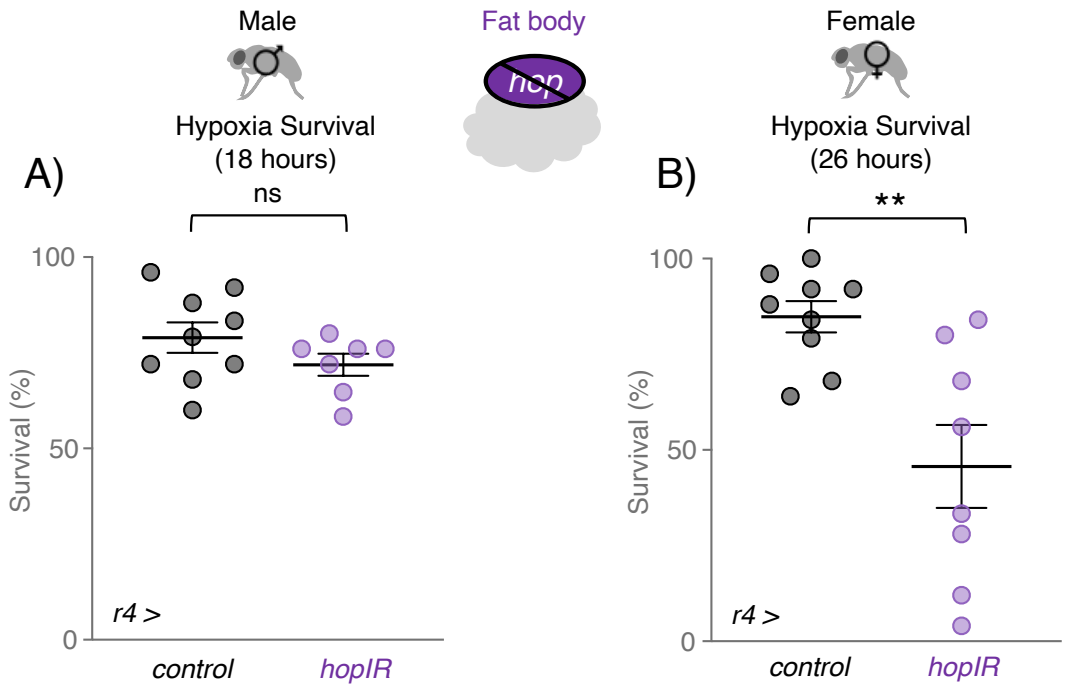

Figure 21. Knockdown of *hopscotch* in the fat body leads to increased sensitivity to hypoxia in adult females, but not males. Control (*r4>control*) and fat body hopscotch knockdown (*r4>hopIR*) adult females and males were placed in 1% O_2 and survival was measured. A) Fat body hopscotch knockdown in male flies show no effect on hypoxia survival. B) Female flies had reduced survival in hypoxia when fat body hopscotch was knocked down. Data represents mean ± SEM, N≥7. ns not significant, **$p < 0.005$, unpaired *t*-test. Data points represent individual samples.

3.11 Upd3 Targets the Fat Body to Mediate Metabolic Changes Involved in Hypoxia Tolerance

The data shown so far has established that the gut induces *upd3* which is required for hypoxia tolerance in adult female *Drosophila.* This gut *upd3* is partially dependent on gut Yorkie

signaling and is negatively regulated by fat body Sima. I next examined potential targets of gut *upd3* signaling in the regulation of hypoxia tolerance.

3.11.1 Hopscotch is Required for Hypoxia Tolerance in Adult Females, Not Males

I was interested in studying which tissue STAT signaling was essential for hypoxia tolerance. To do this, I knocked down Hopscotch, the *Drosophila* Janus Kinase, using various Gal4 drivers in different tissues and looked at survival in hypoxia. One of the tissues I performed this experiment in was the fat body. I used *r4-Gal4* crossed to *UAS hopIR* to generate flies which had knockdown of fat body Hopscotch *(r4>hopIR).* I separated males and females, then exposed them to 18 and 26 hours of hypoxia respectively and counted survival. Interestingly, I found that fat body Hopscotch knockdown *(r4>hopIR)* did not have a significant effect on survival in males (Figure 21A) but did significantly reduce survival in females (Figure 21B), when compared to their sex-matched controls *(r4>control).* This result is consistent with the sexual dimorphism in hypoxia survival with the $upd3^{\Delta}$ null mutants in hypoxia. Furthermore, these results also suggest an Upd3 gut-to-fat body signaling network which might control hypoxia tolerance.

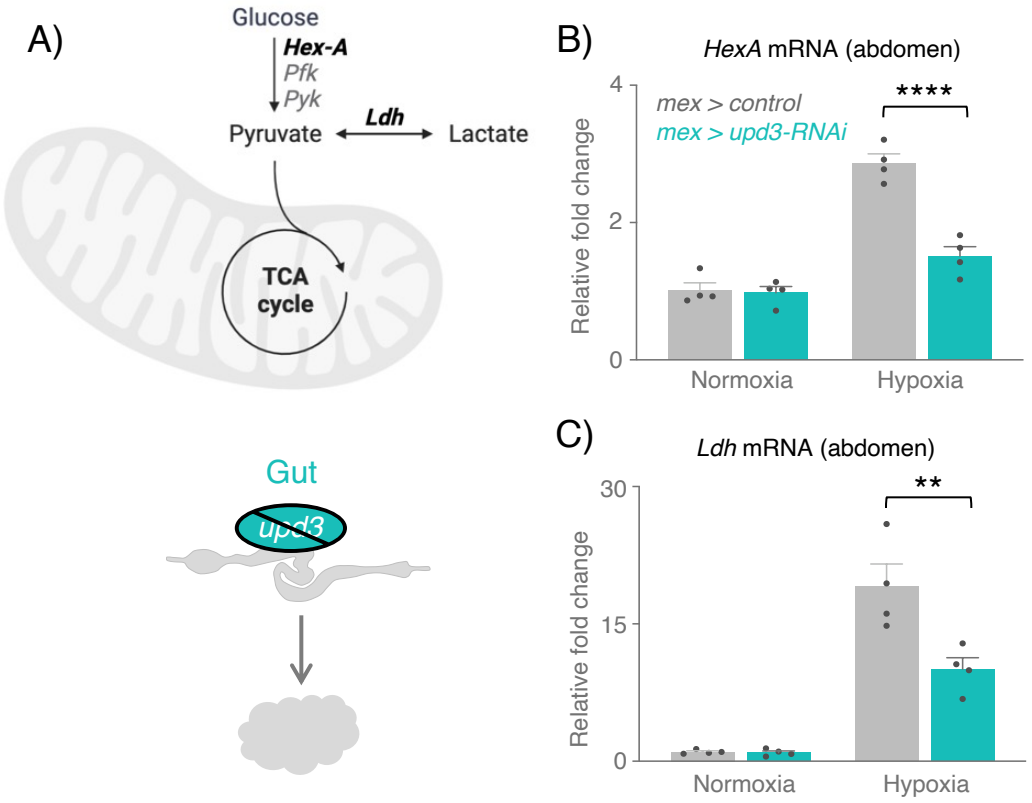

Figure 22. Gut-derived *upd3* is required for induction of glycolytic genes, *HexA* and *Ldh*, in the female fat body upon exposure to hypoxia. Control (*mex>control*) and gut Upd3 knockdown (*mex>upd3-RNAi*) adult females were placed in hypoxia (1% oxygen) for 16 hours and glycolytic genes were measured in dissected abdomens. A) Glycolytic pathway. B) *HexA* and C) *Ldh* mRNA levels were induced in the abdomens of females exposed to hypoxia, which was blocked when gut-derived *upd3* was knocked down. Data represents mean + SEM, N=4. **p < 0.005, ****p < 0.00005, Student's *t*-test following 2-way ANOVA. Data points represent independent samples normalized to *RpS24* mRNA levels.

3.11.2 Gut-Derived Upd3 Acts Non-Autonomously to Control Glycolysis in the Female Fat Body

Next, I aimed to resolve what were the possible functional consequences of gut derived *upd3* signaling on the fat body. The fat body is well-studied and characterized as the central regulator

of metabolism and nutrient sensing (Britton & Edgar, 1998; Colombani et al., 2003). Previous studies from our lab have shown that changes in metabolism, such as glycolysis, altered mitochondrial activity and lipid metabolism, can exert strong non-autonomous effects on animal physiology (Sriskanthadevan-Pirahas et al., 2022; Lee et al., 2019). Therefore, I was interested in measuring metabolic gene expression changes in the fat body of animals where gut *upd3* signaling was blocked. Given the importance of metabolic switching from oxidative phosphorylation to anaerobic glycolysis I focused on expression of glycolytic genes. The glycolytic pathway converts glucose to pyruvate using key enzymes Hexokinase A (*HexA*), phosphofructokinase (*Pfk*), and pyruvate kinase (*PyK*). The anaerobic phase of glycolysis utilizes lactate dehydrogenase (*Ldh*) to maintain redox balance. To determine whether gut *upd3* affects fat body metabolic gene expression, I dissected abdomens, which are enriched for fat body, from animals with gut Upd3 knockdown (*mex>upd3-RNAi)* that were exposed to either normoxia or hypoxia. Controls (*mex>control*) in hypoxia upregulate *HexA* and *Ldh* mRNA levels (Figure 21A, B). This suggests that animals are upregulating anaerobic glycolysis in response to severe oxygen deprivation. Interestingly, gut Upd3 knockdown (*mex>upd3-RNAi*) animals showed a strong suppression of abdomen *HexA* and *Ldh* mRNA levels compared to the controls (*mex>control*) in hypoxia (Figure 21A, B). This suggests that gut *upd3* likely targets the fat body to promote metabolic switching to glycolysis in hypoxia.

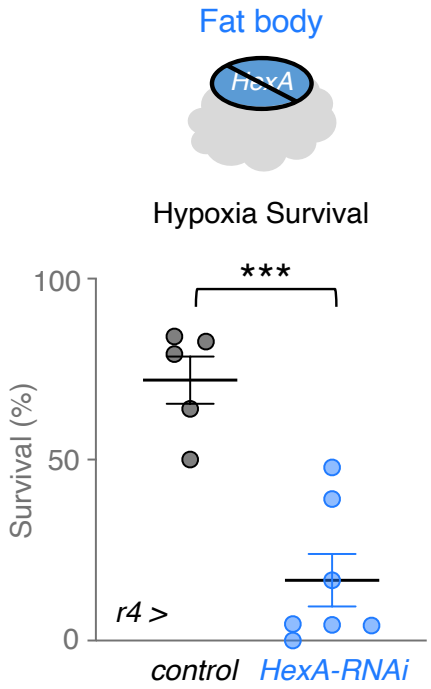

Figure 23. Fat body Hexokinase A is required for female hypoxia survival. Control (*r4>control*) and fat body *HexA* knockdown (*r4>HexA-RNAi*) adults were in hypoxia (1% O_2) for 26 hours and survival was measured. Fat body *HexA* knockdown leads to reduced survival in hypoxia. Data represents mean ± SEM, N=5-7. ns not significant, ***p < 0.0005, unpaired *t*-test. Data points represent individual samples.

3.11.3 Fat Body Hexokinase A is Required for Hypoxia Tolerance in Females

Previous literature shows that the switch from oxidative phosphorylation to glycolysis is an adaptation which is required for animals to tolerate low oxygen conditions. Since I showed that *upd3* controls expression of fat body glycolytic enzymes during hypoxia, I wanted to investigate their requirement in mediating hypoxia tolerance. To do this, I used fat body driver *r4-Gal4* and crossed animals to a *UAS HexA-RNAi* line to generate knockdown of Hexokinase A in the fat body (*r4>HexA-RNAi).* After exposing these animals to hypoxia, I measured survival. I found that fat body Hexokinase A knockdown (*r4>HexA-RNAi)* reduced survival in hypoxia compared

to controls (*r4>control)* (Figure 22). Together, these results demonstrate that glycolytic enzymes are induced in the fat body by gut derived *upd3* and are necessary to mediate hypoxia tolerance.

CHAPTER FOUR: DISCUSSION

Taken together, my results suggest a model in which hypoxia induces *upd3* expression from the *Drosophila* intestine to coordinate glycolysis in the fat body, and that this is specifically required for female hypoxia tolerance (Graphical Abstract, pg. iii). I found that a fine balance of *upd3* signaling was necessary for optimal survival in hypoxia, either too much or too little *upd3* signaling negatively affected tolerance. Induction of gut *upd3* appeared to be partially through the Hippo pathway component, Yorkie, and independent of classic ROS/JNK/p38 signaling. Interestingly, I found a non-autonomous role for the conserved hypoxia transcription factor HIF-1α/Sima in the fat body to restrain excess gut *upd3* levels. Finally, I found that gut derived *upd3* mediates hypoxia tolerance through signaling to the fat body and controlling glycolytic gene expression. This induction of glycolysis is an important adaptation in flies to withstand acute low oxygen exposure. This requirement for JAK/STAT signaling was found to be necessary for female but not male tolerance, suggesting a potential sexually dimorphic requirement for JAK/STAT activation in hypoxia. Below I will discuss these aspects of my model in more detail.

4.1 Fine-Tuning of Upd3 Signaling is Important for Hypoxia Tolerance

In one of my first experiments, I saw that *upd3* was required for female hypoxia survival (Figure 6). Later experiments conversely showed that overexpression of this cytokine signaling impaired ability of flies to tolerate hypoxia (Figure 9). Specifically, *upd3* induction in the intestine was required for survival, and induction of *upd3* was through Yki signaling (Figure 20). Together,

these results give credence to the central theme of my work, which is that *upd3* levels need to be tightly controlled in hypoxia to promote survival – too much or too little *upd3* is deleterious.

My data also suggests there are at least two transcription factors that are important for this fine-tuning – Yorkie and Sima. I saw a decrease in *upd3* induction in the gut when I performed gut *yki* knockdown, however this effect was not complete, at ~30%. This could be a result of incomplete knockdown of the Yki RNAi; therefore, we could try other Yki RNAi lines or do RNAi to Scalloped (homolog of mammalian Tead), the DNA binding partner of Yki. Indeed, Scalloped knockdown was shown to have stronger effects on blunting infection induced *upd3* induction compared to Yorkie knockdown in the initial paper which showed their role as *upd3* regulators (Houtz et al., 2017). It is interesting to speculate as to how hypoxia might induce Yki, which is classically regulated upstream of the Hippo-Warts pathway (homologs of mammalian Mst and Lats family of proteins). These regulate Yki in response to changes in cell shape, cell-cell interactions, and cell polarity (Snigdha et al., 2019). Interestingly, I see that one response to hypoxia is an alteration in the tissue morphology of the gut. Although I did not quantify this, another graduate student in our lab (P. Bodkhe) has recently explored this and has seen that hypoxia leads to shortening of gut length. This could result in changes to cell shape which triggers Yki activation. Furthermore, two papers have described how hypoxia exposure can disrupt cell polarity in *Drosophila* cells (Dong et al., 2015; Lu et al., 2022), so future studies may: i) look to see if hypoxia disrupts enterocyte shape, polarity, cell-cell interactions, ii) use genetic manipulations to see if hypoxia functions through Hippo and Warts to control *upd3.*

Another intriguing result that emerged from my work was that the conserved HIF-1α/Sima transcription factor restrains excess *upd3* cytokine signaling to promote survival in low oxygen (Figures 8 and 17). Even more interesting, these effects were non-autonomous – fat body Sima controls gut *upd3*, suggesting the role of Sima in this case is to limit excess *upd3* induction which causes lethality in hypoxia (Figure 9). The lethal effects of too much *upd3* may be like the cytokine-storm-like response seen in uncontrolled immune signaling in humans. In support of this, I saw that hypoxia lethality of ubiquitous Sima knockdown was partially reversed when I genetically reduced *upd3* levels (Figure 10). Although this effect was not dramatic, this was expected because HIF-1α/Sima plays so many roles in hypoxia, such as promoting tracheation and regulating metabolism, so genetically restoring the negative effect of Sima on *upd3* would likely not fully reverse the lethality seen with whole-body Sima knockdown. My subsequent work pinpointed a role for fat body Sima in the negative regulation of gut *upd3*, therefore we could look to see if fat body Sima knockdown leads to reduced hypoxia survival and whether this is rescued by reducing *upd3* levels. One exciting area for future work will be to explore how Sima in the fat body can negatively regulate gut *upd3*. As HIF-1α/Sima is a transcription factor, it is likely that it restrains *upd3* signaling indirectly by targeting negative regulators of its induction. It is further suggestive that some communication must be occurring, perhaps fat body Sima controls the level of a secreted molecule that signals to the fat body. This molecule could be a classical signaling molecule, such as cytokines or growth factors, or a signaling metabolite. The fat body Sima-regulated signals could control gut Yki signaling or act on whichever mechanisms account for the remaining ~70% induction of *upd3* that is Yki-independent.

4.2 Gut-Fat Body Crosstalk in Hypoxia

Control of homeostasis is reliant the ability for tissues to sense changes in the environment in addition to communication between different tissues. Metabolic homeostasis follows this theme, networks of inter-organ communication coordinate food consumption, nutrient storage, and energy production. Certain tissues can act as sensors of changes in environmental cues, such as changes in nutrient levels, pathogenic infection, or fluctuation in oxygen levels, and then, in response, act as endocrine regulators of whole-body adaptive responses. My work pinpoints a role of the gut as a sensor in hypoxia. This is consistent with the increasingly appreciated function of the gut as a regulator of whole-body health. The gut often acts as the primary sensor of environmental challenges – nutrients, enteric pathogens, ingested toxins – and then can signal to other tissues to trigger systemic adaptation to these environmental cues (Stojanović et al., 2022). The gut is also richly innervated with trachea as a part of its normal morphology, it is ideally poised to serve as an organismal sensor of changes in oxygen levels. If this is the case, the gut can co-opt its endocrine roles to signal changes in oxygen to other tissues.

My studies suggest that one role for gut *upd3* is to control fat body glycolysis to promote hypoxia tolerance (Figures 22 and 23). Several studies have shown evidence for an intestinal-neuronal-fat body signaling axis in which the gut senses dietary nutrients and signals through a neuronal relay to control glycolysis in the fat body (Scopelliti et al., 2019; Koyama et al., 2021). Our lab and others have studied gut-fat body communication in oral infection and demonstrated how this crosstalk is involved in crucial metabolic changes like lipid store depletion to mount

effective immune responses (Zhao & Karpac, 2020; Charroux & Royet, 2022; Deshpande et al., 2022)

In studies of wound healing in mice, an IL-17/HIF-1α axis was discovered to be responsible for promoting glycolysis to promote epithelial migration under injury-induced hypoxic conditions (Konieczny et al., 2022). This study was particularly interesting because it suggests IL-17 is not only upstream of HIF-1α, but is required for optimal HIF-1α activation. Another recent paper on skin wound repair showed how interleukins cooperate with HIF-1α to control metabolic changes needed for regeneration (Liu et al., 2023). Increased glycolytic metabolism is a classic conserved response to low oxygen, of which HIF-1α is the best described mediators. Therefore, one intriguing possibility from my work is that gut *upd3* signals to the fat to augment activation of HIF-1α/Sima, a conserved inducer of glycolytic gene expression and, in turn, HIF-1α activity in the fat body can feedback to control gut *upd3* levels. This signaling axis might act as a rheostat to fine-tune both cytokine signaling and metabolic changes in hypoxia. Future studies could test this by seeing if gut *upd3* and fat body STAT control Sima levels or activity by affecting Sima target genes.

In all, we know that metabolic reprogramming is an important adaptation to environmental challenge. However, we know considerably less about how tissues communicate with each other to coordinate this reprogramming in hypoxia. My work has elucidated a gut-fat body network of signaling in which both tissues communicate with each other to promote hypoxia tolerance by regulation of glycolysis. Future work to further investigate the mechanisms underlying this

regulation, as well as the transduction of signals between tissues would provide valuable insight into whole-organism coordination of response under low oxygen conditions.

4.3 Sexual Dimorphic Requirement of JAK/STAT in Hypoxia Tolerance

One striking finding from my work was that the role for *upd3* in promoting hypoxia tolerance was sex dependent. Although induction of *upd3* and downstream STAT target genes is seen in both males and females exposed to hypoxia, I found a sexual dimorphic requirement for this cytokine in promoting survival whereby *upd3* was required for female, but not male survival (Figure 6). I also showed a requirement for fat body Hopscotch in mediating female, not male, hypoxia tolerance (Figure 21). To our current knowledge, this is the first demonstration of a sexual dimorphic role for *upd3* in the control of physiological and stress response in flies. All other studies on the Upds in flies have reported results in only a single sex, have not mentioned which sex was used, or have used mixed sex populations. In my work, since the induction of the JAK/STAT pathway essentially similar in both males and females, is it unlikely that the differences in survival phenotypes are due simply to lack of pathway activation in either sex. This leaves open several potential explanations.

Classically, sex differences in physiology, metabolism or disease susceptibility have focused on differences in sex hormones as being the underlying cause. However, recent studies, pioneered by work in *Drosophila*, have shed a light on cellular sexual identity as an important influence on disease phenotypes (Ober et al., 2008; Arnold, 2012). Sexual differentiation in *Drosophila* is determined by the number of X chromosomes (Salz & Erickson, 2010). In females carrying two

X chromosomes, the splicing factor *Sex-lethal (sxl)* is expressed and directs cell-autonomous female sexual identity by splicing its target gene the sex determination factor, *transformer (tra),* leading to production of a functional protein, TraF. In male with only one X chromosome, no Sxl is produced, *tra* is not spliced correctly and no functional TraF protein is made. Hence it is the presence of functional TraF protein that cell-autonomously establishes female identity (Boggs et al., 1987; Inoue et al., 1990). Thus, by manipulating expression of Tra in males and females, we can switch the sexual identity of specific tissues or cells – overexpression of TraF in male cells is sufficient to switch their identity to female, which knockdown of TraF is sufficient to switch their identity to male. Recently, researchers have used this approach to generate sexual chimeras to show how the identity of specific cells or tissues explain male-female differences in physiology. For example, in the *Drosophila* intestine, Hudry et al. (2016) found that sexual identity of intestinal stem cells (ISCs) direct tissue homeostasis and regeneration. Interestingly, the same research group had previously described a female-specific midgut remodelling in response to mating to meet metabolic demands associated with reproduction (Reiff et al., 2015). This suggests the female intestine has an inherent greater capacity of plasticity compared to the male intestine due to sexual identity of cell types like the ISCs. Another study using both flies and mice showed that switching the sexual identity of enterocytes (ECs) regulated autophagy to mediate intestinal health and lifespan (Regan et al., 2022). Our lab showed that male-female differences in growth during the larval period are explained by the sexual identity of the fat body which can control systemic insulin signaling (Rideout et al., 2015). Finally, Wat et al., (2021) showed how the sexual identity of a subset of neurons that produce the glucagon-like adipokinetic hormone (Akh) in the fly can determine male-female differences in fat storage.

Based on these studies, there are several interesting experiments which can explore the mechanism underlying the sexual dimorphic requirement for JAK/STAT in females. In my results, I found that manipulation of the *upd3* in the gut (Figure 6) and Hopscotch in the fat body (Figure 21) resulted in sexually dimorphic hypoxia survival. The two tissues related to this sexual dimorphism are the gut and fat body. Using gut or fat body-specific drivers, we can express or knockdown *transformer*^{Female} *(tra*^{F}*).* Masculinization of female cells can be done through *tra*^{F} RNAi and feminization of male cells by expression of *tra*^{F}. In doing so, we could see if switching tissues to female would make male flies susceptible to loss of *upd3* in hypoxia. Likewise, we could see if switching tissues to male would make female flies resistant to loss of *upd3.* Also, since one of my initial observations was that males were more sensitive than females to severe (1% O_2) hypoxia, we can generate sexual chimeras to see whether the sexual identity of any specific tissues(s) can explain the general difference in hypoxia sensitivity between males and females. Performing these kinds of experiments would help support the hypothesis of a sexual dimorphic requirement for JAK/STAT signaling in hypoxia.

4.4 Significance

At its core, my project sought to answer a basic science question, which was: how does systemic hypoxia mediate whole-animal responses? The approach I took, and my findings ultimately extended what we can learn from cell culture studies alone. Give the parallels between fly and human physiology and the conserved nature of cytokine/JAK/STAT signaling, my findings may also have relevance to our understanding of human hypoxia biology in both normal and pathological settings. For instance, my work may be informative to understanding chronic systemic hypoxia in human diseases such as inflammatory bowel disease (IBD), rheumatoid arthritis, and certain cancers which are by caused or exacerbated by the deregulation of hypoxia and inflammatory pathways (Taylor, 2008). Inflammatory bowel disease lends itself to gut hypoxia and so hypoxia-induced cytokine production in this context may be relevant for disease pathology.

My work demonstrates that these immune signals can be co-opted by organisms faced with low oxygen conditions. Inflammation is a necessary stress response to fight pathogens and facilitate wound healing. The normal immune response needs to be carefully regulated, however in conditions where this regulatory balance is lost, excess proinflammatory cytokines can result in a 'cytokine-storm'. This concept of a 'cytokine-storm' has been associated with a variety of human diseases such as graft-versus-host disease, pancreatitis, multiple sclerosis, respiratory infection, and multiple organ dysfunction syndrome (Tisoncik et al., 2012). In the absence of Sima in hypoxia, flies may experience something like a 'cytokine-storm' due to unrestrained Upd3 signaling. This may have important implications in therapies designed to target HIF-1α. In

instances where intratumoral hypoxia or genetic alterations lead to overexpression of HIF-1α, therapies have been developed to inhibit its activity (Semenza, 2003a). However, my results suggest that HIF-1α also plays a role in restraining excess cytokine signaling. Therefore, the implication is such that inhibition of HIF-1α could cause a 'cytokine storm' and lead to worse outcomes overall for patients.

Moreover, I found that gut *upd3* could also result in beneficial systemic responses. For instance, I found that gut *upd3* targets the *Drosophila* fat body to induce glycolysis and promote survival. While we have extensive knowledge about how HIF-1α and glycolysis are important drivers of tumor progression and metastasis, my work is suggestive of the possibility that cytokines and other immune signals could also drive these pathways. A further understanding of the signaling between HIF-1α and immune signals, like IL-6/Upd3, may provide additional therapeutic target options for a range of diseases.

4.5 Caveats, Limitations and Future Directions for My Work

4.5.1 What is the Role of Upd2 in Hypoxia?

In my initial experiments, I found that hypoxia also induces expression of Upd2. I chose to focus on Upd3 in my thesis work mainly because early in my project I identified a requirement for Upd3 in female hypoxia tolerance. Hence, it is still an open question as to whether Upd2 promotes hypoxia tolerance and, if so, whether these effects occur in cooperation with the role of Upd3 that I have discovered.

Most of the published literature on either Upd2 or Upd3 focus on one or the other, with very few investigating the cooperative role they may play in mediating responses to stress. Therefore, it would be interesting to test whether Upd2 is needed for hypoxia tolerance and whether the loss of Upd2 and Upd3 together would give rise to strong phenotypes than knocking down either Upd2 or Upd3 alone. We can also use tissue-specific RNAi or overexpression of Upd2 and Upd3, both alone and together, to test tissue-specific roles for these ligands in hypoxia tolerance. For example, as previously mentioned, all unpaired ligands signal through one common receptor, Domeless, to activate JAK/STAT signaling. The fat body Hopscotch knockdown experiments (Figure 21) showed a requirement for Hopscotch (JAK) in hypoxia survival but it not clear whether this requirement reflects signaling mediated by Upd2 or Upd3, or a combination of both. We can therefore knockdown either or both and use a STAT-GFP transcriptional reporter to see if STAT signaling in the fat body is affected. Conversely, we can mimic the effects of hypoxia and test if acute adult-onset induction of Upd2 and/or Upd3 in the gut is sufficient to stimulate STAT activity in the fat body.

4.5.2 What Mechanisms Explain Full Induction of Gut Upd3 in Hypoxia?

My work found a role for the Yki pathway in activating *upd3* signaling in the female intestine (Figure 20). However, knockdown of Yki only attenuated ~30% of intestinal *upd3* in hypoxia. One study which I used to guide my RNAi screen of candidate regulators of *upd3* (Figures 18, 19, 20) concluded that convergence of multiple pathways (ROS, JNK, p38, Hippo, TGF-β) acts as a genetic network to control transcription of *upd3* in microbial infection. (Houtz et al., 2017). It is possible that this would also be the case in hypoxia regulation of intestinal *upd3.* To test this, I could generate fly lines which either contain RNAi or mutations in multiple combinations of

these candidate pathways and measure *upd3* levels in hypoxia. This could be informative to tell us if more than one of these pathways is required for full induction of *upd3* in hypoxia.

It is also possible that *upd3* levels are being regulated by other transcription factors or signaling pathways. In their study, Houtz et al. (2017) also carried out a combination of *in silico* analysis, yeast one-hybrid screening and functional RNAi screening of all fly transcription factors to identify factors that could directly regulate the *upd3* promoter. This work generated a candidate list of approximately two dozen potential positive and negative regulators of *upd3.* We can therefore carry out a RNAi screen of these factors to see if they mediate *upd3* expression in hypoxia. We can also test other candidate signaling pathways. For example, I showed that the homeodomain-interacting protein kinase (Hipk) is required for low oxygen tolerance (Ding et al., 2022). Interestingly, Hipk is known to directly regulate JAK/STAT signaling as well as Yorkie directly (Tettweiler et al., 2019; Chen & Verheyen, 2012; Poon et al., 2012). In the future, it would be interesting to test knockdown of Hipk and whether it affects *upd3* levels and glycolytic gene expression.

4.5.3 Does Upd3 Have Other Roles in Hypoxia?

In my work, I used survival as a readout for hypoxia tolerance. I found that gut *upd3* is required for female survival in hypoxia (Figure 13) and does so through inducing glycolytic gene expression in the fat body (Figure 22 and 23). While useful in providing insight into genes and pathways required for acute hypoxia tolerance, there are several other phenotypes which might be affected by Upd3 in hypoxia. In fact, Upd3 most likely plays other roles in hypoxia other than signaling to the fat body to control glycolysis. Our lab has shown that larval TAG levels increase

due to increased lipid accumulation in the fat body and that these lipid stores are important for tolerating low oxygen (Lee et al., 2019). Although these experiments were conducted in larvae at a milder 5% O_2 level, it would be interesting to investigate whether 1% O_2 might also remodel lipid droplets in the adult fat body and whether this is dependent on JAK/STAT signaling in any way.

I also found a suppression in female fecundity, the number of viable progeny produced, following acute 1% O_2 exposure (Ding et al., 2022). For females, reproduction is a very energetically expensive process which can be suppressed when animals encounter stress like nutrient deprivation and fungal infection (Drummond-Barbosa & Spralding, 2001; Schwenke et al., 2016). Therefore, animals often exhibit a trade-off in the dedication of resources towards either survival or reproduction depending on available resources and environmental conditions. In direct support of this trade-off hypothesis, sterile females which cannot produce eggs have enhanced ability to tolerate infection (Short et al., 2012). It is possible that there are pathways induced in hypoxia which are responsible for the reallocation of resources away from reproduction, towards promoting survival. RNAseq analysis showed that genes involved in egg laying and chorion shell formation were found to be downregulated in hypoxia (Ding et al., 2022), therefore it would be interesting to ask whether this occurs in an Upd3 or JAK/STAT-dependent manner to promote overall survival.

As previously mentioned, a canonical role for *upd3* specifically in the gut is to regulate intestinal stem cell renewal. Exhaustion of this stem cell pool has been correlated with reduced lifespan by breakdown of gut epithelial layer integrity. Therefore, it remains to be explored whether an acute

burst of intestinal *upd3*, a stimulator of ISC proliferation, in hypoxia could lead to stem cell dysplasia and contribute to decreased survival.

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