UNIVERSITY OF CALIFORNIA, SAN DIEGO

# The Role of Notch in Survival During Chronic Hypoxia

A dissertation submitted in partial satisfaction of the requirements
for the degree of Doctor of Philosophy

in

Biology

by

DeeAnn Williams Visk

Committee in charge:

Professor Gabriel Haddad, Chair
Professor Randall Johnson, Co-Chair
Professor Amy Kiger
Professor William McGinnis
Professor Jason Yuan
Professor Dan Zhou

2011

The Dissertation of DeeAnn Williams Visk is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2011

# DEDICATION

*To my family*

*Deloris, my Mom, who always encouraged all my endeavors;*

*Wayne, my dad;*

*Chuck, my beloved husband;*

*and*

***especially, my children, RuthAnn and Kenny,***

*who accompanied Mommy to the lab on many Saturdays*

EPIGRAPH

*Hope lies in dreams, in imagination and in the courage of those who dare to make dreams into reality.*

*--Jonas Salk*

*[Engraved on the steps to the entrance of the Salk Institute]*

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LIST OF ABBREVIATIONS

NICD……Notch Intracellular Domain

CNS……Central Nervous System

EN……+/+;Eaat1GAL4/Eaat1GAL4;UAS-NICD/UAS-NICD

GFP……Green Fluorescent Protein

CVD……Cardio-Vascular Disease

Eaat1……high affinity glutamate transporter 1

UAS-GAL4……Upstream Activating Sequence-Galatose responsive gene 4

GAL4……..Galatose responsive gene 4

PDH……pyruvate dehydrogenase

MARCM……Mosaic Analysis with a Repressive Cell Marker

T-ALL……T Acute Lymphoblastic Leukemia

PTEN……Phosphatase and tensin homolog

NF-κB……Nuclear Factor-Kappa B

RNAi……..RNA inference

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## VITA

1985-1989 Bachelor of Science, Michigan State University

1991-1996 Research Technologist, University of Washington

1996-1999 Caregiver for daughter

1999-2003 Research Associate, University of California, San Diego

2003-2011 Doctor of Philosophy, University of California, San Diego

## PUBLICATIONS

"Experimental selection of hypoxia-tolerant Drosophila melanogaster." Zhou D, Udpa N*, Gersten M*, Visk DW*, Bashir A, Xue J, Frazer KA, Posakony JW, Subramaniam S, Bafna V, Haddad GG. *Proceedings of the National Academy of Sciences*, 2011 vol 108 no 6, p2349-54, 2011. *All authors contributed equally.

"The asparaginyl hydroxylase factor inhibiting HIF-1alpha is an essential regulator of metabolism". Zhang N, Fu Z, Linke S, Chicher J, Gorman JJ, Visk D, Haddad GG, Poellinger L, Peet DJ, Powell F, Johnson RS. *Cell Metabolism*, vol 11 no 5, p364-78, 2010.

"*Drosophila*, a golden bug, for the dissection of the genetic basis of tolerance and susceptibility to hypoxia." Dan Zhou, **DeeAnn W. Visk**, Gabriel G. Haddad. *Pediatric Research*, vol 66 no 3, p239-247, 2009.

"Targeting of TAK1 by the NF-KB protein Relish regulates the JNK-mediated immune response in *Drosophila*." Jin Mo Park, Helen Brady, Maria Grazia Ruocco, Huaiyu Sun, **DeeAnn Williams**, Susan J. Lee, Tomohisa Kato Jr., Normand Richards, Kyle Chan, Frank Mercurio, Michael Karin, and Steven A. Wasserman. *Genes & Development*, vol 18, p 584-594, 2004.

"Analysis of muscle creatine kinase regulatory elements in recombinant adenoviral vectors." Michael A. Hauser, Ann Robinson, Dennis Hartigan-O'Conner, **DeeAnn Williams-Gregory**, Jean N. Buskin, Steve Apone, Christopher J. Kirk, Stephen Hardy, Stephen D. Hauschka, and Jeffery S. Chamberlain. *Molecular Therapy*, vol 2 no1, p 16-25, July 2000.

"Harvest protocol to reduce variability of soluble enzyme yield from cultured cells." Jean N. Buskin, **DeeAnn L. Gregory**, William LaFramboise, and Stephen D. Hauschka. *BioTechniques*, vol 20 no 1, p 92, January 1996.

FIELDS OF STUDY

**Hypoxia using *Drosophila* as a model organism** 2005-2011, Dr. Gabriel G. Haddad, UCSD.

**Innate Immunity and RNA regulation of male fertility factors**, 2001-2003, Dr. Steven A. Wasserman, UCSD.

**Tissue culture models of Abl knockout mice**, 1998-1999, Dr. Jean Wang, UCSD.

**Muscle-specific gene regulation utilizing tissue culture of mouse muscle cells**, 1991-1996, Dr. Stephen D. Hauschka, University of Washington, Seattle.

ABSTRACT OF THE DISSERTATION

The Role of Notch in Survival During Chronic Hypoxia

by

DeeAnn Williams Visk

Doctor of Philosophy
University of California, San Diego, 2011

Professor Gabriel Haddad, Chair

Damage caused by lack of oxygen is a key factor in many serious disease states ranging from stroke and heart attack to asthma and high altitude mountain sickness. Determining the pathways that can ameliorate injuries caused by hypoxia is key to developing treatments and prevention of hypoxic damage. This dissertation examines the role of Notch signaling in potentiating *Drosophila* survival under chronic low oxygen conditions.

Pathway-level analysis of micro-array data of hypoxia-selected flies, the Notch signaling pathway was significantly up-regulated. Using the UAS-GAL4 system, the temporal and spatial specificity of Notch signaling was determined. The most robust results were found with glia-specific GAL4 drive, hence they were chosen for further characterization. Expression patterns of the GAL-4 glial drivers were confirmed by crossing them to a UAS-GFP transgenic fly line.

Immunohistochemical analysis confirmed NICD (Notch Intracellular Domain) over-expression in glia cells. The developmental importance of Eaat1 glia was shown by crossing them to the UAS-reaper line; the results were embryonic lethal. Transcriptional up-regulation of Notch signaling was demonstrated by crossing flies stably over-expressing NICD with a reporter stock.

To address the question of what mechanism Notch utilizes to potentiate survival during hypoxia, several hypotheses were considered. First, Notch signaling may allow Eaat1-expressing glia to form a stem cell niche. This niche, in turn, allows the replacement of damaged/dead neurons injured during hypoxia. Alternatively, other pathways may mediate the survival of these glial cells, which have been shown to be necessary to the survival of the whole organism. The metabolism of these glia could be altered via pyruvate dehydrogenase (PDH). Akt and NF-κB are also known to interact with the Notch pathway. Perhaps canonical downstream target genes of Notch signaling, such as *m-α* act as mechanism to allow survival of these crucial glia and thus the whole organism. To study Notch interacting with other pathways, flies stably over-expressing NICD under the Eaat1 driver were crossed to UAS-RNAi flies targeting genes involved in pathways known to potentiate survival during hypoxia. Both Relish and m-α act downstream of Notch signaling to mediate survival, demonstrating novel means of Notch up-regulation leading to hypoxic survival.

# INTRODUCTION

Hypoxia is defined as a lower than normal level of oxygen. The damage caused by several major diseases such as heart attacks, strokes, asthma, and high elevation is mostly due to lack of oxygen to affected tissues. These various disease states lead to much death and disability throughout the world. According to the Center for Disease Control National Vital Statistics for 2009 heart disease, chronic lower respiratory disease, and cerebral vascular disease are the first, third, and fourth cause of death respectively,(Kochanek et al., 2011). Understanding hypoxic damage various leads to two strategies to develop therapeutic/preventive methods: to study mechanism of damage and block them; or to identify tolerance mechanism and to evoke them. We followed the second strategy. "The total direct and indirect cost of CVD [cardio-vascular disease] and stroke in the United States for 2010 is estimated at $503.2 billion" (Lloyd-Jones et al., 2010). Given the aging population of the United States and ever increasing cost of healthcare, basic research in understanding tolerance to hypoxia is imperative. This work done in this dissertation will allow a better understanding of mechanism that potentiate survival during hypoxic insult and build a foundation for therapies that can invoke tolerance to low oxygen levels.

Hypoxia may be acute, chronic or intermittent. Acute hypoxia is a sudden and immediate lack of oxygen as experienced in a stroke or heart attack. Chronic hypoxia, lower oxygen levels over a long period of time, is experienced during emphysema or lone term asthma. Intermitment hypoxia is seen with sleep

disorders like sleep apnea. These different hypoxic paradigms lead to different responses to low oxygen; hypoxia response is paradigm and duration dependent. This concept must be taken into consideration in the development of experimental protocols. This dissertation studies the effects of chronic hypoxia in the context of whole organism development.

The organism employed to study this phenomena is *Drosophila melanogaster*. Commonly known as fruit flies, *Drosophila* have been scientifically studied for over a century. Genetically malleable with many well characterized tools to manipulate gene expression, *Drosophila* are relatively inexpensive and simple to culture and maintain. With short generation time and large number of progeny, this organism lends itself to straightforward studies. Additionally, many pathways and human diseases are successfully modeled in *Drosophila* (Zhou et al., 2009). Hence, a simple but powerful model organism—*Drosophila*—can be brought together to yield important insights into the understanding of hypoxia.

# CHAPTER 1: NOTCH SIGNALING PATHWAY

Discovery of signaling pathways commenced over a hundred and thirty years ago with the study of simple, single-celled organisms. Glycolysis was first studied by Pasteur in 1879 using yeast that ferment beer (Pasteur et al., 1879). He showed that fermentation required living organisms, and that specific end products were derived from fermentation by these organisms. These metabolic pathways of these simple organisms are evolutionarily conserved from lower order organisms--such as yeast and bacteria--through vertebrates.

More complex organisms have a variety of systems such as nervous, skeletal, and digestive. While the types of pathways conserved from single-celled organisms through vertebrates are often metabolic in nature, in multi-cellular and multi-system organisms such as *Drosophila*, developmental pathways are also conserved through vertebrates. Hence, lower order organisms, such as *Drosophila* and *Caenorhabditis* have been successfully used to elucidate complex pathways during development, such as the pathways that regulate development. One pathway conserved from flies to humans is the Notch pathway, which is implicated in neural development in both species (delaPompa et al., 1997).

The Notch system is simpler in *Drosophila* than in mammals, lending itself to easier study with fewer components. This can be seen in Table 1.1 below, comparing the genes in the classic *Drosophila* Notch signaling pathway with those found in humans. Some genes have one to one homologous in flies and

humans, such as *O*-fucosyltransferase, which is involved in proper processing of nascent Notch. There are also those with four different homologues of Delta, a Notch ligand, in humans compared to flies.

**Table 1.1. Comparison of homologous genes in humans and flies in the Notch signaling pathway.**

| ***Drosophila* gene** | ***Homo* homologues** |
|---|---|
| *Notch* | *Notch-1*<br>*Notch-2*<br>*Notch-3*<br>*Notch-4* |
| *fringe* | *Lunatic Fringe*<br>*Radical Fringe*<br>*Maniac Fringe* |
| *O-fucosyltransferase* | *O-fucosyltransferase* |
| *delta* | *Delta-1*<br>*Delta-2*<br>*Delta-3*<br>*Delta-4* |
| *serrate* | *Jagged-1*<br>*Jagged-2* |
| *Supressor of Hairless [Su(H)]* | *Recombination signal Binding Protein for immunoglobulin kappa J region (RBPJ)* |

The name, Notch, originated when a mutant fly with a “notch” in its wings was observed and propagated in 1917 (Morgan, 1917). From this initial observation, many other mutants affecting the notch in *Drosophila* wings were

discovered and characterized. From these humble beginnings, the Notch signaling pathway revealed itself.

In succeeding years, Notch was further characterized utilizing classic genetic techniques. As the fields of molecular biology and cell biology advanced, many new tools became available for biologist to study *Drosophila*. With these new techniques, Notch signaling pathway became better understood.

Developmentally, Notch signaling is traditionally involved in lateral inhibition or boundary formation. An example of lateral inhibition is found in the development of sensory organ precursor cells. To determine cell fate (e.g. neurons or glia), Notch signals from the one cell to those adjacent, preventing the surrounding cells from becoming neurons. Boundary formation involving Notch is found in *Drosophila* wing development where the margins of the wings are delineated by Notch signaling.

Classically, the Notch signaling pathway works by ligand recognition of Delta or Serrate. This results in extracellular cleavage of Notch and ligand, followed by intracellular cleavage of Notch mediated by the γ-Secretase complex. The Notch Intracellular Domain (NICD) then translocates to the nucleus and acts as a transcriptional co-activator. In the last several years, Notch signaling has been implicated in non-developmental, non-canonical signaling. As mentioned in the above review,

> One of the greatest challenges in studying Notch signaling is the inability to predict the outcome of Notch activation, owing to its multiple roles. The canonical Notch pathway is strikingly simple; there are no second messengers. How is it, then, Notch signaling can have such pleiotropic effects? One mechanism is through

> extensive crosstalk with other pathways. It is generally accepted that Notch signaling integrates multiple pathways, thereby tuning downstream expression to fit the cell's current needs and situation, that is, the cell's context…(Ables et al., 2011).

Several genome-wide screens have found several other purposes for Notch signaling that range from metabolism to endocytic links (Mourikis et al., 2010; Mummery-Widmer et al., 2009; Saj et al., 2010). Perhaps, it is because Notch has been so intensely studied for so long, that such an astonishing number of pathways was found. With further study of newer pathways, the same number and diversity of connections will be found.

In 2005, the finding that Notch is necessary to prevent the differentiation of neural stem cells was published (Gustafsson et al., 2005). Notch signaling was found to be necessary to maintain undifferentiated cultures of muscle and neural cells during hypoxia. Additionally, hypoxia itself was seen to increase Notch signaling. Further work in human embryonic stem cells corroborates this idea (Androutsellis-Theotokis et al., 2006; Prasad et al., 2009).

In addition to the strengths of utilizing *Drosophila* as a model organism, adult *Drosophila* withstands acute anoxia amazingly well. When subjected to no oxygen for several hours, adult flies quickly stop moving, fall over and remain motionless. When ambient atmospheric gases are reintroduced, the fly typically begins to move again. An adult *Drosophila* recovered from this anoxic treatment appears to eat, fly, mate, and produce viable progeny just as any normal fly (Haddad and Ma, 2001).

Several approaches have been employed by the Haddad Lab to study

hypoxia using *Drosophila*. They include mutagenesis screens utilizing ionizing radiation, ethylmethlysufonate, and P-element insertions to generate mutations. Also, P-element insertions of enhancer/promoters that can be driven with the UAS-GAL4 system have been used in over/under-expression screens. Intriguingly, a new method of studying changes in genetic material by employing natural selection of flies via hypoxia has also proved fruitful in dissecting genetic changes that permit better survival during chronic hypoxia.

In the Haddad lab studies of *Drosophila* in hypoxia showed that blocks to development under hypoxia take place at two major points in development. The first developmental block prevents hatching of the embryo into the first instar larval stage. The second block impedes eclosion, when the pupae evacuate the pupal case and emerge as adults.

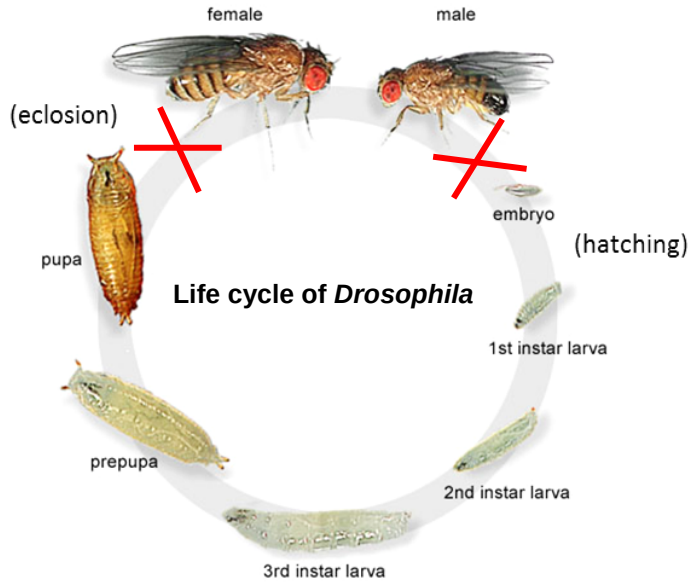



**Figure 1.1. Blocks to *Drosophila* development during low oxygen. Red "X" indicates developmental blocks during chronic hypoxia.**

Beginning February 5, 2002, the Haddad lab began selection of flies under lower and lower oxygen levels over many generations to generate a hypoxia-tolerant *Drosophila* model. To ensure a genetically diverse background, 27 isogenic lines, isolated from the wild, were crossed *en masse*, and the F1 was subjected to chronic low-oxygen levels (Zhou et al., 2007). A schematic of this can be found in Figure 1.2. Additionally, Dr. Zhou has shown that these changes are genetic (as opposed to a physiological adaptation) by growing the hypoxia-selected flies from generation F47 at normoxia for 8 generations, and then returning them to 5% oxygen, where, unlike naïve flies, the majority of them matured to adulthood.

As a control, the 27 lines mentioned above were crossed *en masse* and reared in the same type of chambers in parallel, but under normoxic conditions. At each generation, samples of both control and hypoxia-selected flies were collected as embryos, third instar larvae, and adults. The adult and third instar larvae where analyzed with cDNA micro-arrays at generation F18.

The resulting data showed that many more genes were differentially expressed in larvae than in adults, as seen in Graph 1.1. Coupled with the hypoxic block to eclosion, third instar larvae were chosen as the developmental time point to be studied in this dissertation. During the 3$^{rd}$ instar stage of development, Drosophila larvae attain enough energy reserves to metamorphosis into adults during pupation. The continued development of several organ systems such as the brain and gut involves the proliferation of cells after a period of quiescence following hatching (Hartenstein et al., 2008; Mathur

et al., 2010). Thus, the role of Notch in 3rd instar larvae presents an added dimension of development in these organs.

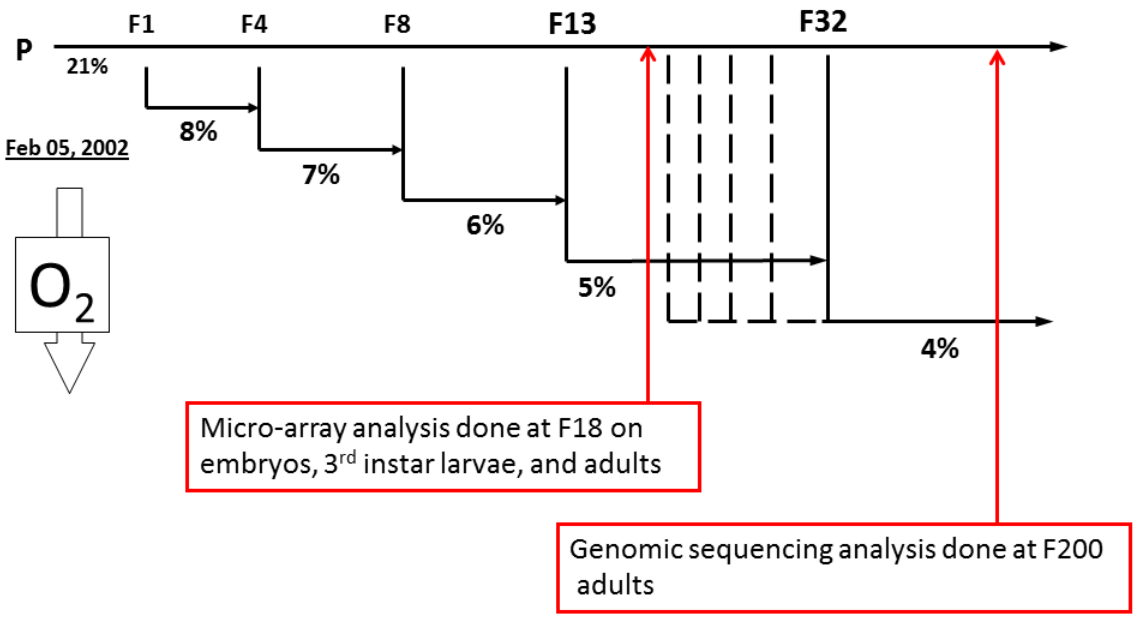
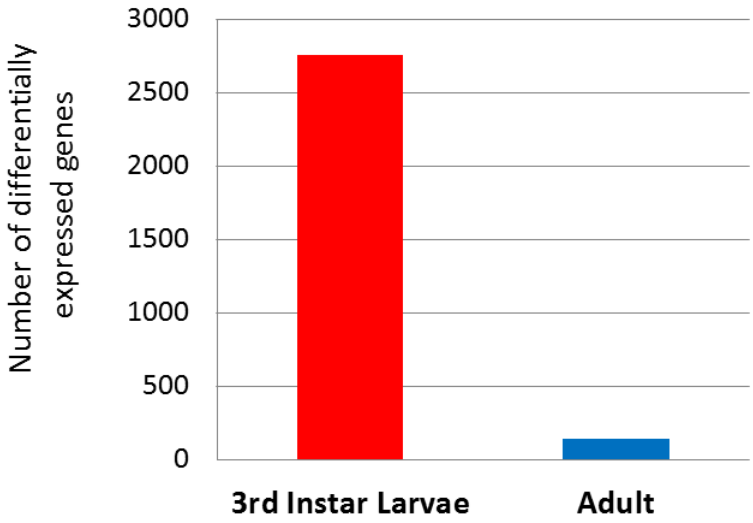



**Figure 1.2. Hypoxia-Selection of *Drosophila* and Experimental Analysis.**

Interpretation of microarray can easily be confounded by assuming that the long lists of genes differentially expressed fluctuate without regard to each other. "Changes essentially resulting in long lists of genes that were found to have significantly changed in transcript levels are assessed by microarray analysis on an individual basis, transcript levels. However, in biology these changes do not occur as independent events as such lists suggest, but in a highly coordinated and interdependent manner" (Werner, 2008). This pitfall of micro-array data analysis can be avoided by looking at differentially expressed pathways as opposed to individual genes. This yielded many pathways differentially expressed in hypoxia-selected flies as compared to the controls, as seen in Table 1.2 below.

**Graph 1.1. Number of differentially expressed genes in 3rd instar larvae and adults (hypoxia-selected vs. control).**




The Notch signaling pathway was chosen for several reasons. A very low P-value for this pathway analysis indicates that the Notch pathway is highly relevant. Many elements of the Notch pathway are well characterized and the pathway is well conserved from flies through humans. Notch is one of the key pathways involved in regulating development. Additionally, previous works link the necessity of Notch signaling to survival during hypoxia. Table 1.2, shown below, lists the genes in the Notch pathway that are differentially regulated in the microarray. Details of how, when, and where in the development of a whole organism the up-regulation of the Notch signaling pathway potentiates survival, however, remain to be elucidated.

The Notch signaling pathway begins with the proper folding and glycosylation of nascent Notch by O-fucosyltransferase and Fringe. The processed Notch is then inserted into the cell membrane; upon binding of a ligand (Delta or Serrate) the γ-Secretase complex, of which Nicastrin and Anterior

**Table 1.2. Pathway differentially expressed pathways based on Haddad lab microarray anlysis.**

| Name of pathway | P-value | Number of Genes Changed | Total Number of Genes in Pathway |
|---|---|---|---|
| Role of IAP-proteins in apoptosis | 0.000564 | 6 | 36 |
| Notch signalling pathway | 0.000878 | 6 | 39 |
| Caspase cascades | 0.000951 | 7 | 54 |
| Role of APC in cell cylce regulation | 0.002916 | 4 | 34 |
| Role of SUMO in p53 regulation | 0.002933 | 5 | 21 |
| Role of Akt in hypoxia induced HIF1 activation | 0.003264 | 6 | 50 |
| Ligand-dependent transcription of retinoid-taget genes | 0.003707 | 10 | 125 |
| Pten pathway | 0.005271 | 6 | 55 |
| Gylcolysis and Gluconeogenesis part 2 | 0.005752 | 3 | 13 |
| Receptor-mediated HIF regulation | 0.005976 | 5 | 40 |
| PIP3 signaling in cardiac myocytes | 0.006789 | 7 | 76 |
| EGFR signaling via PIP3 | 0.007492 | 4 | 27 |
| Insulin receptor signaling pathway | 0.008141 | 5 | 43 |
| Role of AP-1 in regulation of cellular metabolism | 0.008141 | 5 | 43 |
| WNT signaling pathway, part1, degradation of beta-catenin in the absence of WNT signaling | 0.008542 | 4 | 28 |

Pharynx Defective(APH) are component proteins, the Notch Intracellular Domain (NICD) is released where it translocates to the nucleus and acts as a transcriptional co-activator with Suppressor of Hairless [Su(H)]. Some downstream targets of Notch include the Enhancer of Split complex genes of *m*-

**Table 1.3. Notch pathway genes differentially expressed in the microarray. Most of these genes are up-regulated and only *suppressor of deltex* is down-regulated.**

| Name of *Drosophila* gene | Fold-increase | Conserved in humans as |
|---|---|---|
| *Suppressor of deltex* | 0.62 | *WW domain containing E3 ubiquitin ligase 1 (Nedd4 ubiquitin ligase family)* |
| *fringe* | 1.55 | *radical fringe, manic fringe, and lunatic fringe* |
| *anterior pharynx defective 1* | 1.63 | *APH-1A* and *APH-1B* |
| *E(spl) region transcript mβ* | 1.64 | *HES4* |
| *bearded* | 1.65 | --- |
| *nicastrin* | 1.72 | *Nicastrin* |
| *Enhancer of split* | 1.81 | --- |
| *E(spl) region transcript mγ* | 1.92 | *HES2* |
| *E(spl) region transcript mδ* | 2.20 | *HES1* |
| *E(spl) region transcript m4* | 2.21 | --- |
| *E(spl) region transcript mα* | 2.27 | --- |
| *O-fucosyltransferase 1* | 2.74 | *Protein O-fucosyltransferase 1* |

*α*, *m-γ*, *m-δ*, *m-4*, *E(spl)*, and *bearded*, which were also up-regulated in the micro-array (Schweisguth, 2004). Finally, Suppressor of Deltex [Su(dx)], is an E3 ubiquitin ligase for NICD. Thus, if *Su(dx)* is down-regulated, the net effect will be an increase in Notch signaling (Sakata et al., 2004). A representation of this

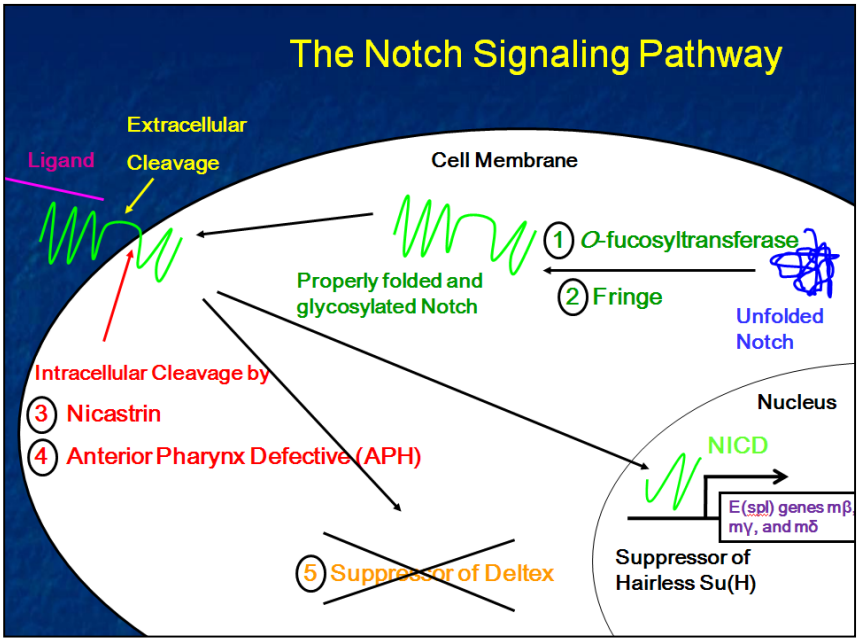



**Figure 1.3. The Notch signaling pathway in relation to genes up-regulated in micro-array**.

can be found in the figure below.

*O*-fucosyltranferase adds a fucose to threonines and/or serines in the EGF region of Notch as well as acting as a chaperone for nascent Notch (Okajima et al., 2008b). Fringe acts on the fucosylated residues of Notch by adding an *O*-glucose to those residues already fucosylated by *O*-Fut1. These modifications result in modulation of Notch interactions with its ligands Serrate and Delta, localization of Notch at adherent junctions, and endocytosis of Notch receptors (Okajima et al., 2008a).

Nicastrin and Anterior Pharynx Defective 1 are part of the γ-Secretase complex that releases the Notch Intracellular Domain after ligand binds to the extracellular portion of Notch. The gene *anterior pharynx defective* was originally found in *C. elegans*; it is a component of the γ-Secretase complex and is abbreviated *aph-1* in *Drosophila* (Schweisguth, 2004). Both APH-1 and Nicastrin are important in stabilizing the holoenzyme Presenilin (Psn) before it has become enzymatically active (Fortini et al., 2004). Nicastrin has also been shown to function in recognition of the cleaved Notch ectodomain and presenting the Notch intracellular domain (NICD) to the γ-Secretase complex for intracellular cleavage (Shah et al., 2005). Interestingly, APH1A, the human gene homolog for *anterior pharynx defective*, is regulated by HIF1-α (Wang et al., 2006). Both Nicastrin and APH1A are necessary for the proper localization of Presenilin, another component of the γ-Secretase complex. Hence, an increase in the amount of APH-1 and Nicastrin increases the -secreatase activity necessary to activate Notch Signaling (Kopan and Goate, 2002).

The enhancer of split genes, *E(spl) region transcript mβ*, *E(spl) region transcript mγ*, and *E(spl) region transcript mδ*, are all basic helix-loop-helix (bHLH) transcription factors that are canonical targets of Notch (Stifani et al., 1992). They act as transcriptional repressors of genes required for neurons to differentiate, such as *achaete* and *scute* (Sriuranpong et al., 2002). The remaining enhancer of split genes, *E(spl) region transcript m4* and *E(spl) region transcript mα* are part of the bearded gene family, as is the gene *bearded*, of course.. The increase in expression level of these Notch target genes shows that there is indeed an increase in Notch signaling.

Thus, from both the literature and experimental evidence in the Haddad lab, the Notch signaling pathway showed promise in developing an understanding of hypoxia resistance. Some questions remain, such as when and where Notch expression increases hypoxic survival remain to be illuminated. The next chapter of this dissertation will address these questions. The third chapter will examine possible mechanisms for Notch-mediated hypoxic survival.

# CHAPTER 2: NOTCH OVER-EXPRESSION POTENIATES SURVIVAL DURING HYPOXIA

A key question in elucidating the role of Notch during hypoxic survival is when and where up-regulation of the Notch signaling pathway leads to an increase in survival. While tissue culture models have provided invalable information on the link between Notch and hypoxic survival, there are few studies in whole organisms. In the context of intact animal models, the question invariably rises of in what tissue(s) expression takes place and during what developmental time period. To address this question spatial and temporal specificty can be determined using the UAS-GAL4 system in *Drosophila* (Phelps and Brand, 1998). Over-expressing the Notch intracellular domain (NICD) increases Notch signal; this idea can be used to selectively increase Notch signal in particular cells.

In the UAS/GAL4 system, a DNA construct containing the Upstream Activating Sequence (UAS) regulatory DNA with the gene of interest—in this example (Figure 2.1) Green Fluorescent Protein (GFP)—is used to generate a UAS-gene-of-interest transgenic fly. A second DNA construct containing a Regulatory Element (RE) which confers temporal and spatial specificity to expression of the GAL4 gene, is employed to generate a second transgenic fly. Hence, when these transgenic flies are crossed to one another, the offspring will express the gene-of-interest in the expression pattern of the Regulatory Element. The gene-of-interest could also be a hairpin loop leading to a Ribonucleic Acid

inhibitory (RNAi) effect in *Drosophila*. Thus, the UAS-GAL4 system does not always have to be over-expression of a gene, or expression of a non-endogenous gene, but can also allow inhibition of a gene of interest

To determine when and where NICD over-expression leads to better survival during chronic hypoxia, a number of GAL4 fly lines were crossed to the UAS-NICD fly line and progeny were assayed for survival during chronic hypoxia. Initial experiments looked only at eclosion rate; later ones, designed to further delineate the most robustly surviving crosses from those less so, also included an adult post-eclosion survival metric. Figure 2.2 shows the experimental design.

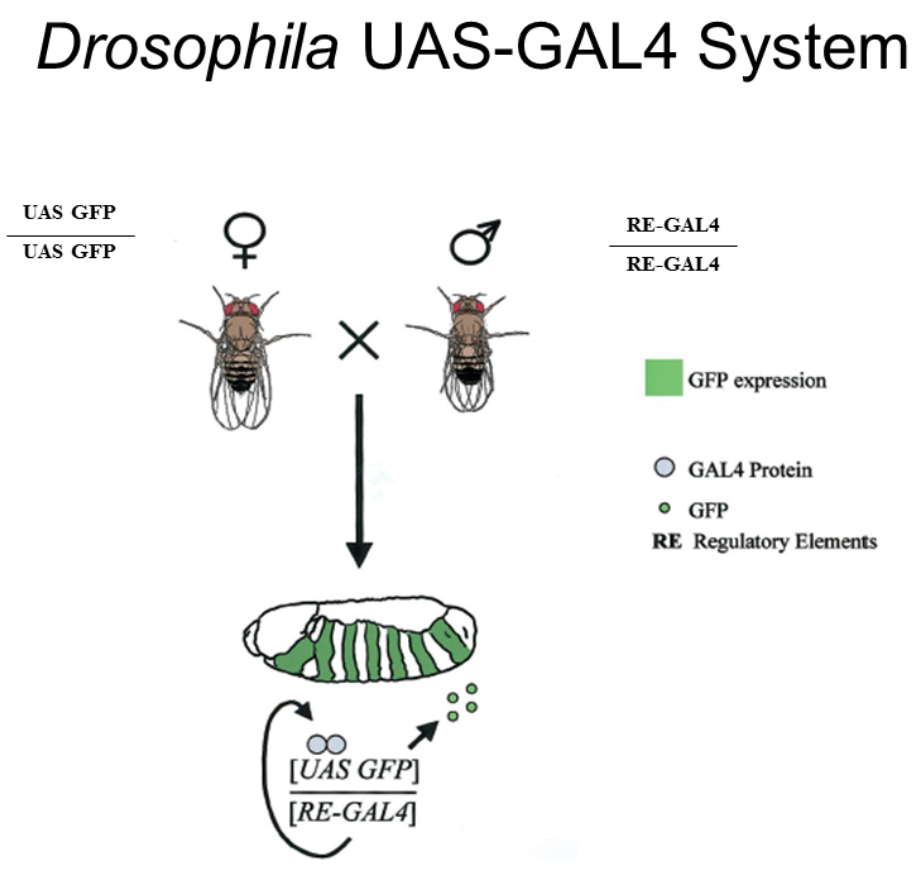



**Figure 2.1. Representation of UAS-GAL4 system of gene expression in *Drosophila*.**

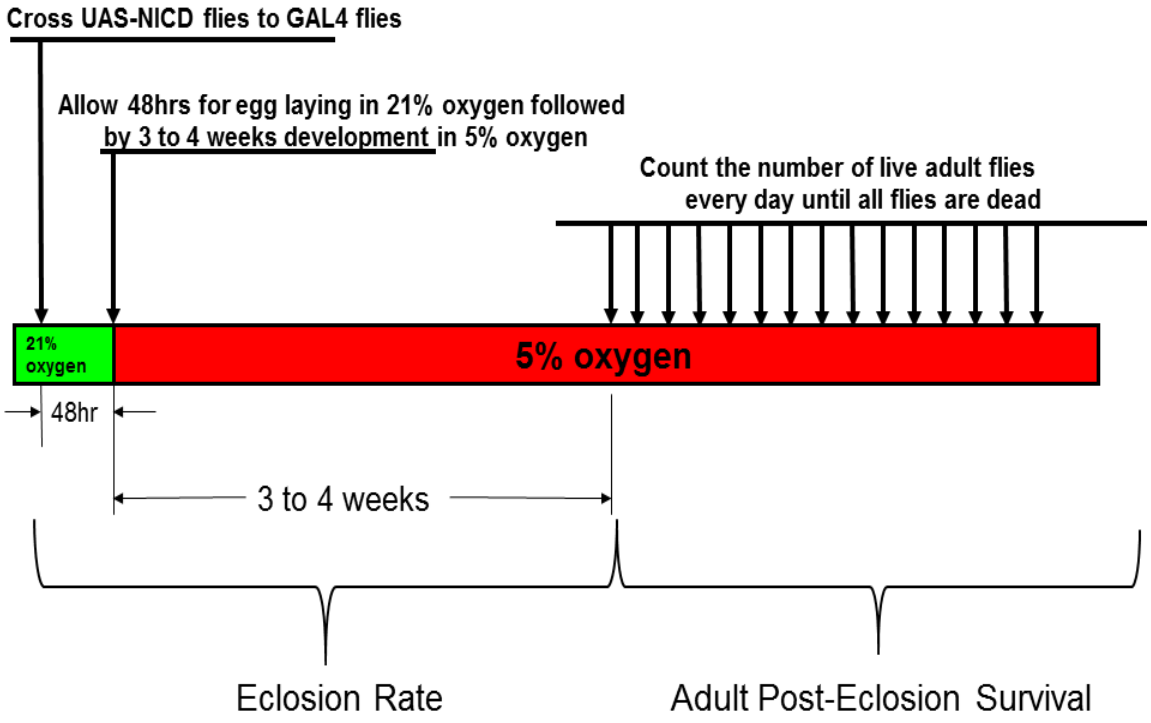



**Figure 2.2. Experimental paradigm used to study survival of Drosophila in low oxygen. For full details, see Appendix 1: Methods and Materials. Briefly, three to five day old UAS-NICD virgin females were crossed to GAL4 males and allowed to mate and lay eggs for 48 hours in ambient conditions then moved to 5% oxygen. Once adults eclosed in 5% oxygen, they were moved to a new vial once a day. The Adult Post-Eclosion rate was determined by counting the total number of surviving adults, including newly eclosed ones.**

Table 2.1 lists the GAL4 lines used in combination with the UAS-NICD fly to assess the potential of Notch over-expression to increase survival during chronic hypoxia. These drivers cover a wide range of cell types from glia and neurons to fat bodies and chrondrotal organs. The expression pattern varies from a very few cells to pan- organism expression, such as in the *daughterless (da)* gene expression pattern.

The results of crossing these GAL4 drivers to UAS-NICD and assaying for eclosion rate and post-eclosion adult survival are shown in Table 2.2. The results show a large range of responses from embryonic lethality to hypoxic

survival comparable to normoxic survival.

**Table 2.1. List of GAL4 drivers selected to be tested in the experimental paradigm: all lines are homozygous, viable, and fertile.**

| GAL4 Driver Name | Fly Base ID for Stock | Expression Pattern |
|---|---|---|
| P{w[+mC]=Eaat1-GAL4.R}2 | FBst0008849 | glial cells that produce the glutamate transporter EAAT1[2] |
| P{GawB}17A | FBst0008474 | glia (embryonic stage 16 to adult) and cardia[2] |
| P{GAL4-Eh.2.4}C21 | FBst0006301 | eclosion hormone-expressing neurons |
| P{GAL4-elav.L}3 | FBst0008760 | nervous system |
| P{RN2-GAL4}P | FBst0007473 | RP2, aCC, and pCC neurons |
| P{Pdf-GAL4.P2.4}2 | FBst0006900 | ventrolateral neurons of the brain and a small number of cells in the CNS |
| P{EcR.GET-BD-GAL4}1 | FBst0005901 | EcR-A-expressing neurons destined for apoptosis at metamorphosis, also in imaginal discs |
| P{GawB}SG18.1 | FBst0006405 | antennal olfactory receptors neurons and processing centers in CNS, also imaginal precursors |
| P{GawB}GII5 1 | FBst0007031 | embryonic peritracheal cells and pericardial cells |
| P{GawB}60IIA | FBst0007029 | nervous system |
| P{CQ2-GAL4}O | FBst0007466 | U/CQ neurons |
| P{GawB}OK107 | FBst0000854 | mushroom bodies |
| P{GawB}109(2)80 | FBst0008769 | multiple dendritic neurons oenocytes and chordotonal organs |
| P{GawB}AB1 | FBst0001824 | salivary gland |
| P{GawB}332.3 | FBst0005398 | amnioserosa and salivary glands |
| P{GawB}Tab2[201Y] | FBst0004440 | primarily in mushroom body |
| P{ato-GAL4.3.6}14a | FBst0006480 | atonal pattern in brain external sensory organ precursor cells |
| P{Lsp2-GAL4.H}3 | FBti0018531 | third instar fat body |
| P{GAL4-sli.S}3 | FBst0009580 | embryonic midline glial cells and MP1 neurons |
| P{GawB}OK307 | FBst0006488 | giant descending neuron circuit |
| P{GawB}DJ752 | FBst0008182 | adult brain muscles and cardia |
| P{GawB}7B | FBst0007365 | brain strong expression in the alpha and beta lobes of the mushroom body weaker expression in alpha', beta', and gamma lobes |
| P{Eip71CD-GAL4.PC}TP1-1 | FBst0006871 | larval epidermis and in brain cells starting at the mid-third instar transition |
| P{GawB}D42 | FBst0008816 | motor neurons |
| P{Ddc-GAL4.L}4.3D | FBst0007010 | dopaminergic and serotonergic neurons |
| P{da-GAL4.w[-]}3 | FBst0008641 | pattern of the da gene |
| P{Ddc-GAL4.L}4.36 | FBst0007009 | dopaminergic and serotonergic neurons |

Not surprisingly, when NICD is expressed under the control of a strong, ubiquitous driver such as the *daughterless* (*da*) gene, the result is embryonic lethal; there is no hatching of the egg to the first instar. In other cases, no pupae

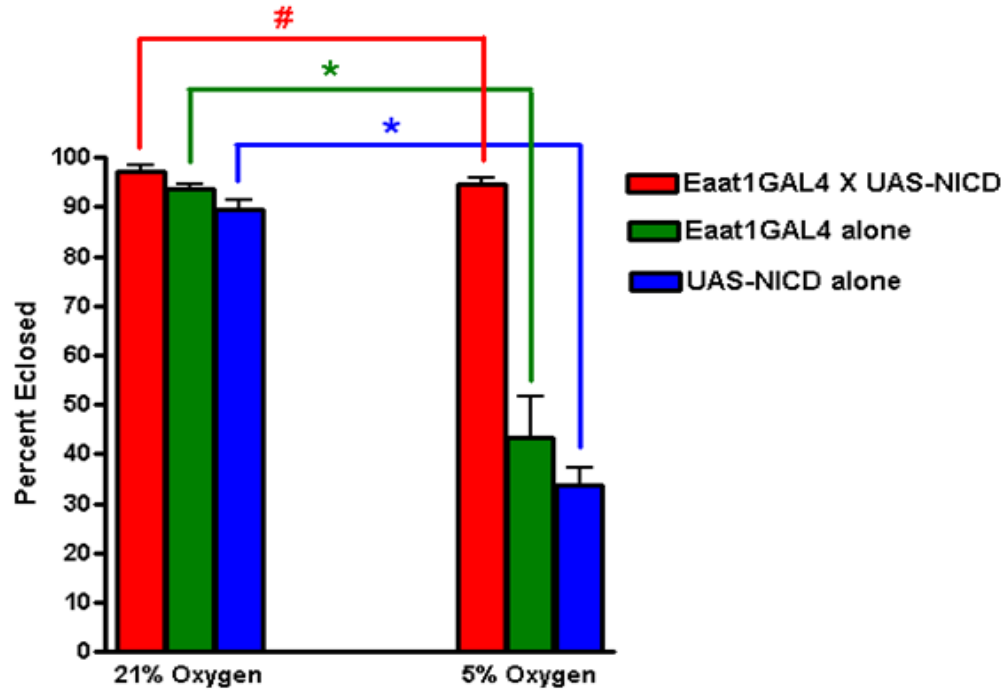
**Table 2.2. Results of crossing selected GAL4 drivers to UAS-NICD and assaying for eclosion and adult post-eclosion survival in 5% Oxygen. For full details, see Appendix 1: Materials and Methods. [#]verified in this dissertation with crosses to UAS-GFP line FBst0006451. All other expression patterns as reported by the Bloomington Stock Collection; [$]Pupation, but no eclosion; [%]No pupae, so not applicable; [*]Embryonic lethal**

| GAL4 Driver Name | Expression Pattern | Percent Eclosed in 5% Oxygen | Adult Survival Post-Eclosion |
|---|---|---|---|
| P{w[+mC]=Eaat1-GAL4.R}2 | glial cells that produce the glutamate transporter EAAT1[#] | 95 | excellent |
| P{GawB}17A | glia (embryonic stage 16 to adult) and cardia[#] | 92 | excellent |
| P{GAL4-Eh.2.4}C21 | eclosion hormone-expressing neurons | 94 | good |
| P{GAL4-elav.L}3 | nervous system | 91 | good |
| P{RN2-GAL4}P | RP2, aCC, and pCC neurons | 90 | good |
| P{Pdf-GAL4.P2.4}2 | ventrolateral neurons of the brain and a small number of cells in the CNS | 82 | good |
| P{EcR.GET-BD-GAL4}1 | EcR-A-expressing neurons destined for apoptosis at metamorphosis, also in imaginal discs | 25 | good |
| P{GawB}SG18.1 | antennal olfactory receptors neurons and processing centers in CNS, also imaginal precursors | 74 | fair |
| P{GawB}GII5 1 | embryonic peritracheal cells and pericardial cells | 69 | fair |
| P{GawB}60llA | nervous system | 62 | fair |
| P{CQ2-GAL4}O | U/CQ neurons | 58 | fair |
| P{GawB}OK107 | mushroom bodies | 55 | fair |
| P{GawB}109(2)80 | multiple dendritic neurons oenocytes and chordotonal organs | 49 | fair |
| P{GawB}AB1 | salivary gland | 69 | poor |
| P{GawB}332.3 | amnioserosa and salivary glands | 53 | poor |
| P{GawB}Tab2[201Y] | primarily in mushroom body | 29 | poor |
| P{ato-GAL4.3.6}14a | atonal pattern in brain external sensory organ precursor cells | 26 | poor |
| P{Lsp2-GAL4.H}3 | third instar fat body | 22 | poor |
| P{GAL4-sli.S}3 | embryonic midline glial cells and MP1 neurons | 14 | poor |
| P{GawB}OK307 | giant descending neuron circuit | 12 | poor |
| P{GawB}DJ752 | adult brain muscles and cardia | 5 | poor |
| P{GawB}7B | brain strong expression in the alpha and beta lobes of the mushroom body weaker expression in alpha', beta', and gamma lobes | 6 | poor |
| P{Eip71CD-GAL4.PC}TP1-1 | larval epidermis and in brain cells starting at the mid-third instar transition | 0[$] | NA[%] |
| P{GawB}D42 | motor neurons | 0[$] | NA[%] |
| P{Ddc-GAL4.L}4.3D | dopaminergic and serotonergic neurons | 0[*] | NA[%] |
| P{da-GAL4.w[-]}3 | pattern of the *da* gene | 0[*] | NA[%] |
| P{Ddc-GAL4.L}4.36 | dopaminergic and serotonergic neurons | 0[*] | NA[%] |

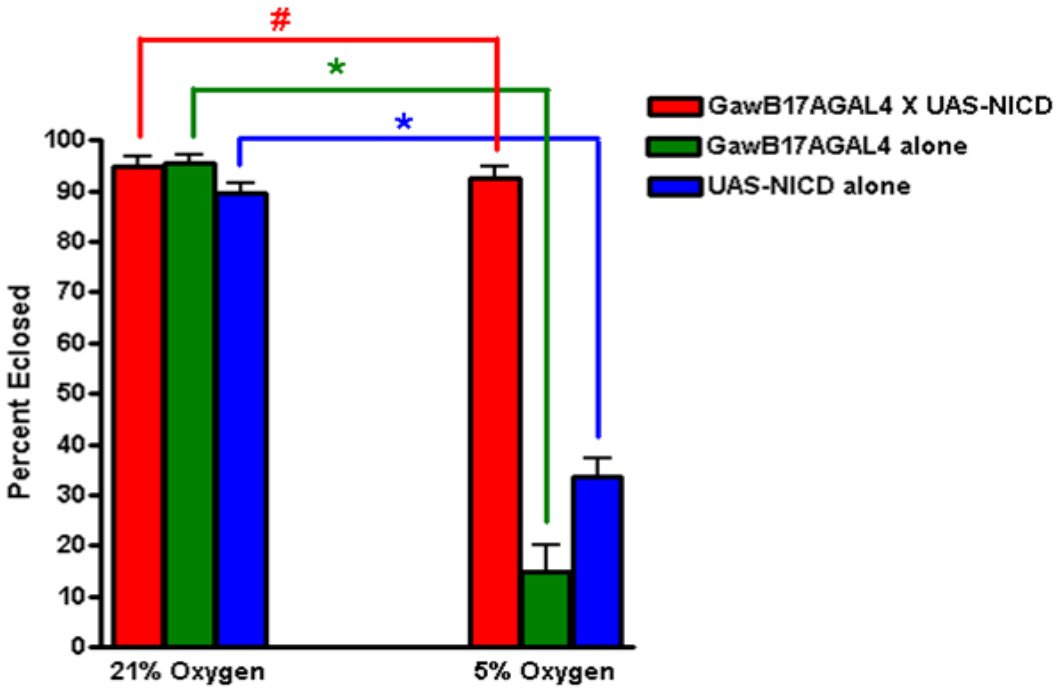
were formed (as seen in the GAL4 driver D42), although eggs hatched. Almost all progeny that pupated but did not eclose appear as fully formed adults within the pupal case (pharate adults).

Fascinatingly, the top two performers on both the eclosion rate and adult post-eclosion were expressed in glia (Rival et al., 2004). The 17A GAL4 driver also expresses in the cardia (which serves as a valve in insects, regulating food passage into the midgut) as well, however, when procardia alone was tested, it had little increase in survival--see GAL4 driver P{GawB}DJ752 in Table 2.2. A closer look at the two highest scoring GAL4 drivers, both expressed in glia of the central nervous system (CNS), reveals the data summarized in the following Graphs (2.1, 2.2, 2.3, and 2.4). While other GAL4 drivers gave good results, these two glia-specific drivers gave spectacular results, greatly increasing survival during hypoxia. Given the low p-values, and similar results of both glial drivers, these two lines were selected for further study. The next question asked was about the spatial specificity (or cell-type specificity) of NICD over-expression. Was the expression solely in glia, or also other cell types? Careful analysis of confocal sections of 3$^{rd}$ instar larval brains revealed that the majority of cells over-expressing NICD under the control of Eaat1 were in repo-expressing glial cells. Repo is a protein used to mark glia cells in *Drosophila*; it labels all glia except midline glia. Neurons are labeled with an antibody against Elav. The NICD antibody also stains endogenous NICD as well as over-expressed NICD transgene. In both cases, the majority of cells over-expressing NICD are glia, while some of the cells are positive for neither Repo nor Elav. With the 17A

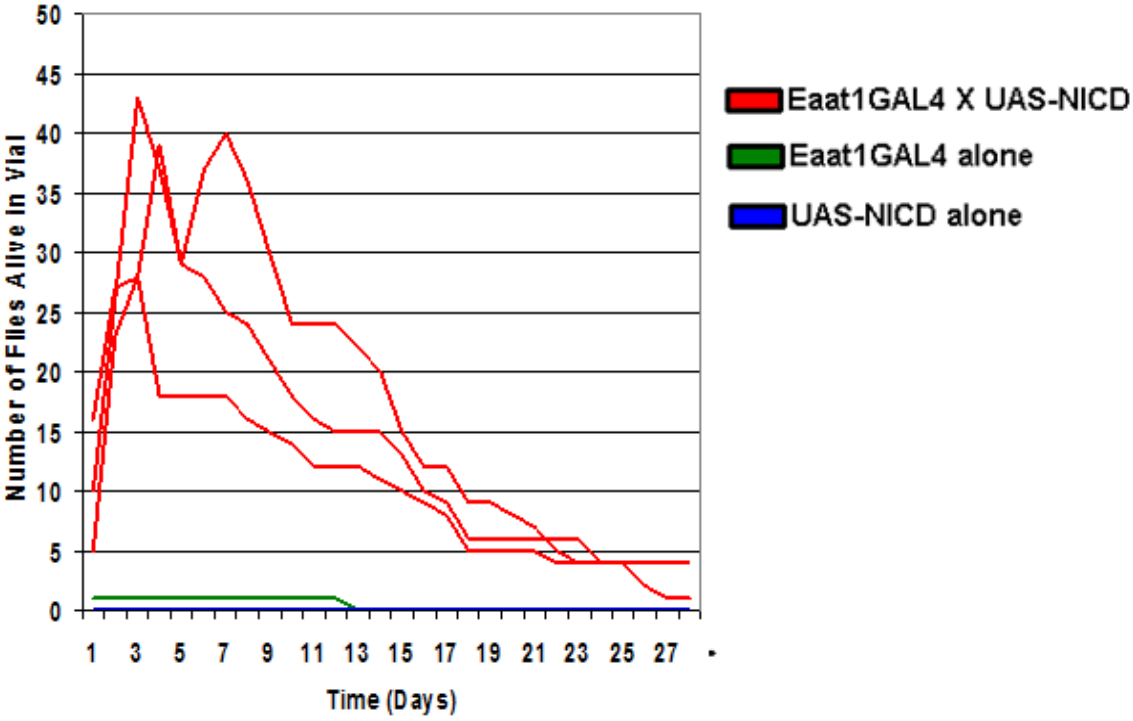
**Graph 2.1. Eclosion rate of Drosophila over-expressing NICD in Eaat1 glia compared to parental controls. Bars equal mean +/- SEM. # = p = 0.205; *= p < 0.001. P-values determined by unpaired t-test.**



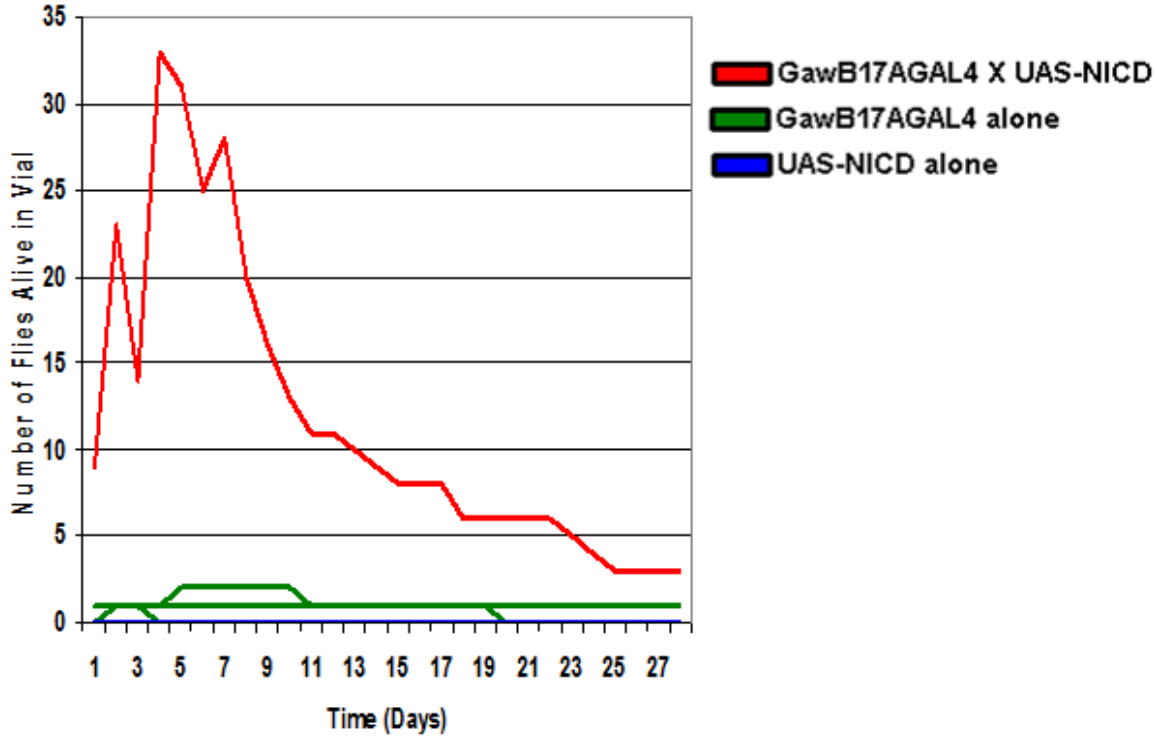

**Graph 2.2. Eclosion rate of Drosophila over-expressing NICD in 17A glia compared to parental controls. Bars equal mean +/- SEM. # = p = 0.456; *= p < 0.001. P-values determined by unpaired t-test.**



**Graph 2.3. Adult survival post-eclosion of Drosophila over-expressing NICD in Eaat1 glia compared to parental controls.**




**Graph 2.4. Adult survival post-eclosion of *Drosophila* over-expression NICD in 17A glia compared to parental controls.**



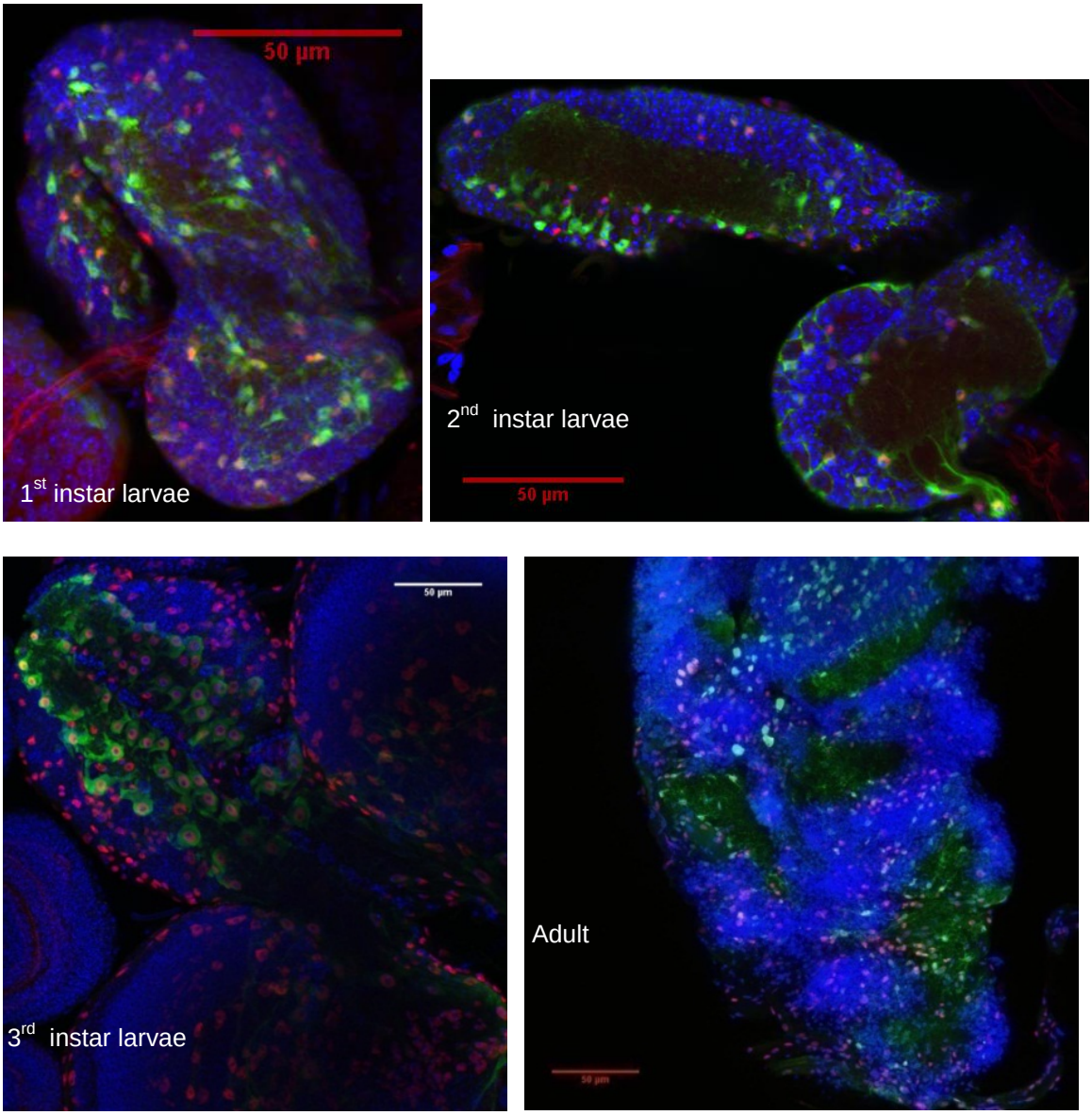



**Figure 2.3. The Eaat1-GAL4 driver was crossed to the UAS-GFP reporter fly line and the resulting progeny were assayed for expression of GFP (green), Repo (red), and DAPI (blue). Z-projections of 5 to 12 slices; scale bar=50µm.**

GAL4 driver, there are a few cells positive for both Elav and NICD.

To better understand when and if Eaat1 glia are essential for the survival of the whole organism, Eaat1-GAL4 flies were crossed to a UAS-reaper line, leading to death of those cells via apoptosis. This cross leads to apoptosis of cells that express Reaper in the pattern of the GAL4 driver. The results again

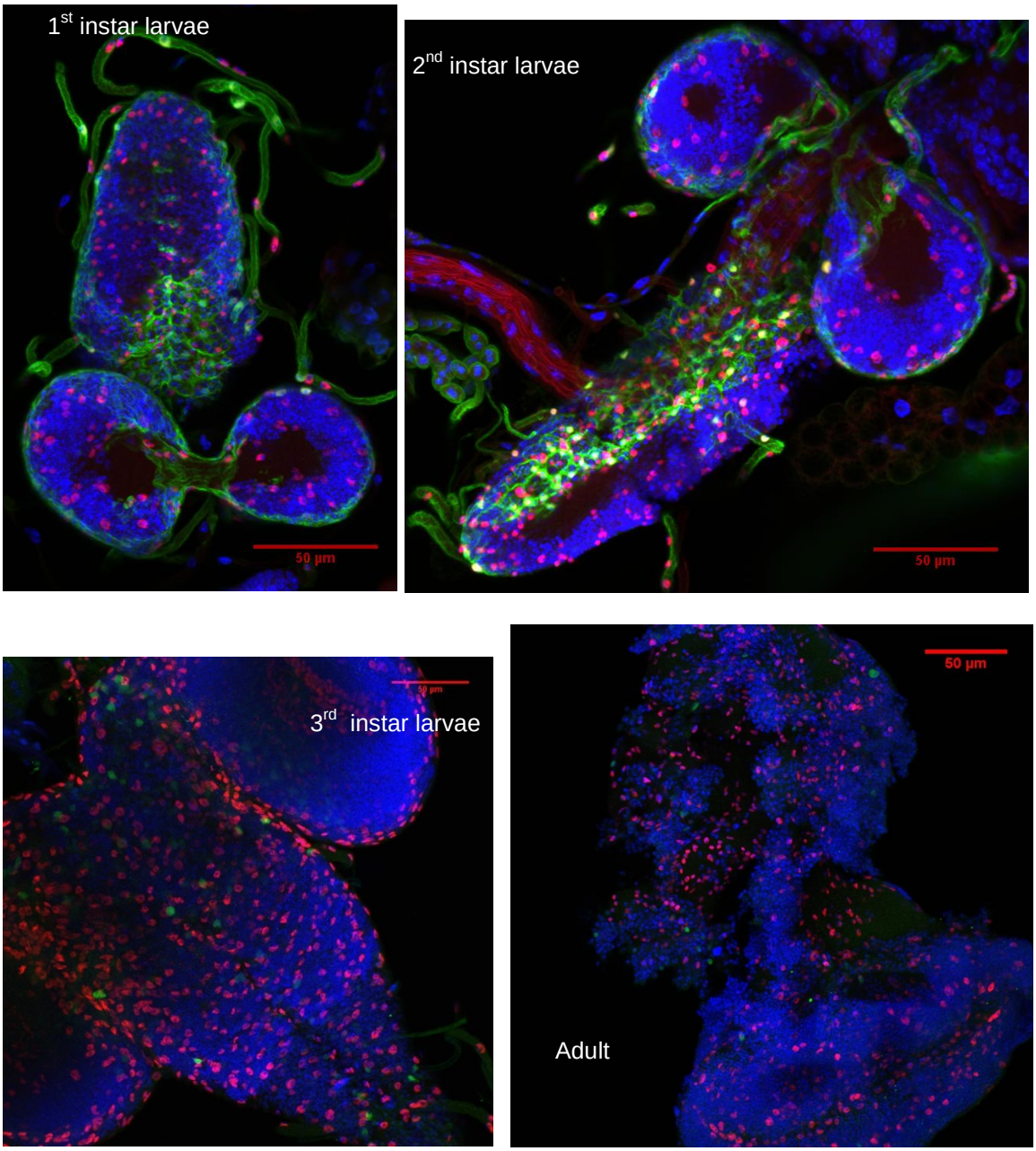
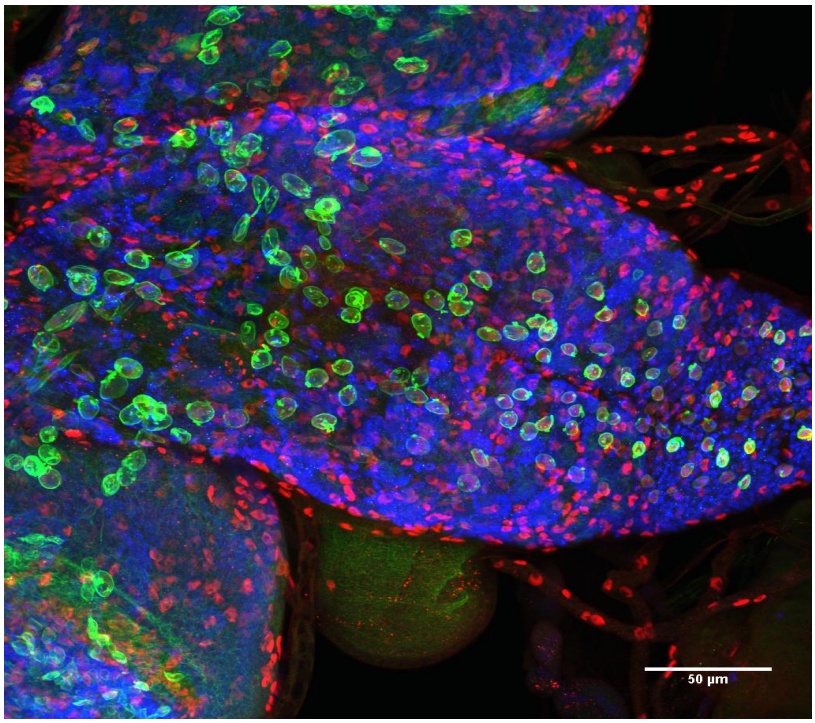



**Figure 2.4. The 17A-GAL4 driver was crossed to the UAS-GFP reporter fly line and the resulting progeny were assayed for expression of GFP (green), Repo (red), and DAPI (blue). Z-projections of 5 to 12 slices; scale bar=50µm.**

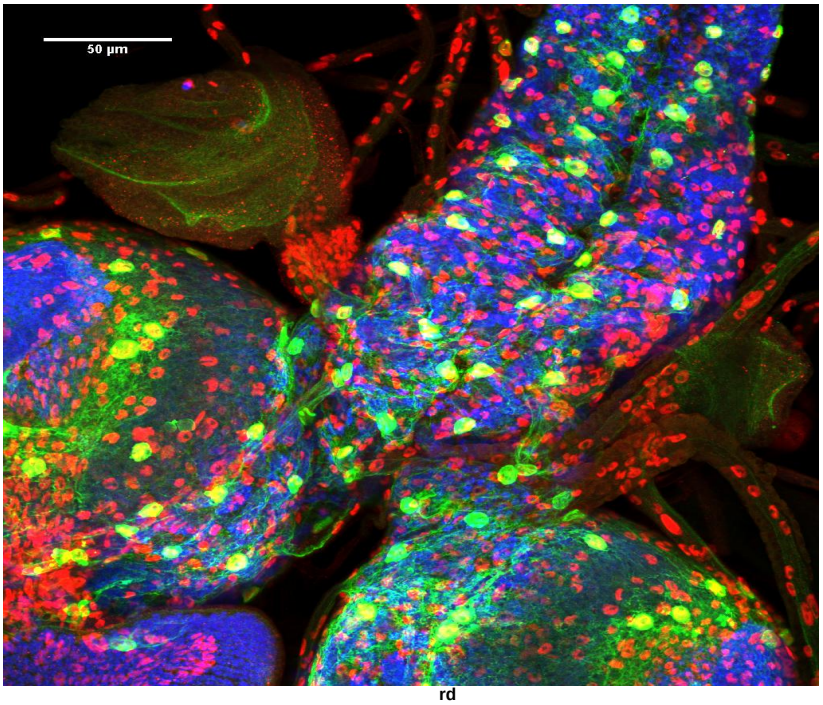
reiterate those of previous publications, showing that the death of Eaat1 cells is embryonic lethal to the entire organism . As an internal control, the second cross shows that flies without the UAS-reaper construct are viable in this background.




Red = Repo (glia)
Green = NICD
Blue = Elav (neurons)

70% NICD$^{+}$ Repo$^{+}$
0% NICD$^{+}$ Elav$^{+}$
30% NICD$^{+}$ Repo$^{-}$ Elav$^{-}$

**Figure 2.5. Eaat1GAL4>UAS-NICD 3 instar brains stained for Repo, NICD, Elav then analyzed with confocal microscopy. Shown is a z-projection of 29 slices. Scale bar = 50 microns.**




Red = Repo (glia)
Green = NICD
Blue = Elav (neurons)

90% NICD$^{+}$ Repo$^{+}$
6% NICD$^{+}$ Elav$^{+}$
4% NICD$^{+}$ Repo$^{-}$ Elav$^{-}$

**Figure 2.6. 17AGAL4>UAS-NICD 3rd instar brains stained for Repo, NICD, Elav then analyzed with confocal microscopy. Shown is a z-projection of 27 slices. Scale bar = 50 microns.**

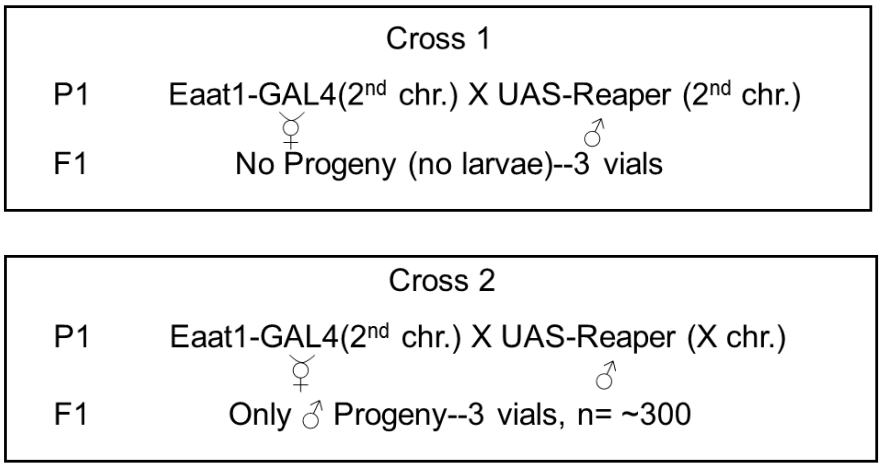



**Figure 2.7. Results of crossing flies with the Eaat1-driven GAL4 transcription factor to the UAS-reaper flies.**

To ask more questions about the characterization of NICD-mediated hypoxic survival, a double balanced fly line containing the Eaat1-GAL4 construct as well as UAS-NICD was derived following the crosses in Figure 2.7. This line

**Let N = UAS-NICD on 3rd chromosome**
**E = Eaat1-GAL4 on 2nd chromosome**
**Sb = TM3 balancer with Stubble marker on 3rd chromosome**
**CyO = CyO balancer on 2nd chromosome**
**Ap = Apterous, mitten-shaped wing marker, 2nd chr., T (2;3) ap [Xa], ap [Xa]**

**The 1st (X) chromosome will be ignored, since it does not carry DNA of interest**

**Step1**

| | | | |
|---|---|---|---|
| **P1** | **+/+;N/N** | **X** | **Ap/CyO;+/Sb** |
| **F1 select for only CyO and Sb flies** | **+/CyO;N/Sb** | **X** | **+/CyO;N/Sb (self)** |
| **F2 select for only CyO flies** | **+/CyO; N/N** | | |

**Step2**

| | | | |
|---|---|---|---|
| **P1** | **E/E;+/+** | **X** | **Ap/CyO;+/Sb** |
| **F1 select for only CyO and Sb flies** | **E/CyO;+/Sb** | **X** | **E/CyO;+/Sb (self)** |
| **F2 select for only Sb flies** | **E/E;+/Sb** | | |

**Step3**

| | | | |
|---|---|---|---|
| **P1 (F2 from Crosses 1 and 2)** | **E/E;+/Sb** | **X** | **+/CyO;N/N** |
| **F1 select for only CyO and Sb** | **E/CyO;N/Sb** | **X** | **E/CyO;N/Sb (self)** |
| **F2 self the F1 for balanced stock** | **E/CyO;N/Sb *or* E/E;N/Sb *or* E/CyO;N/N *or* E/E;N/N** | | |

**NICKNAME: EN line**

**Figure 2.8. Derivation of a *Drosophila* line stably over-expressing NICD under the control of Eaat1-GAL4 driver.**

that stably over-expresses NICD can then be crossed to other UAS lines, allowing the testing of other pathways that interact with Notch. Additionally, this line can be used to address other questions.

To further characterize the over-expression of NICD, the question of the precise subcellular localization arose. To address this question, the EN line was crossed to lines containing inserts of GFP in particular proteins (Quinones-Coello et al., 2007). The first image of Figure 2.9 is that of GFP expressed in protein TER94, which in known to localize with the Golgi, does not co-localize with NICD over-expression. Also in Figure 2.8, GFP inserted into the protein calrecticulin, which is known to localize to the endoplasmic recticulum, does co-localize with NICD over-expression. Thus, the confocal images show that the over-expressed NICD co-localizes not with the endoplasmic reticulum or Golgi, but with the nuclear envelope protein, Lamin.

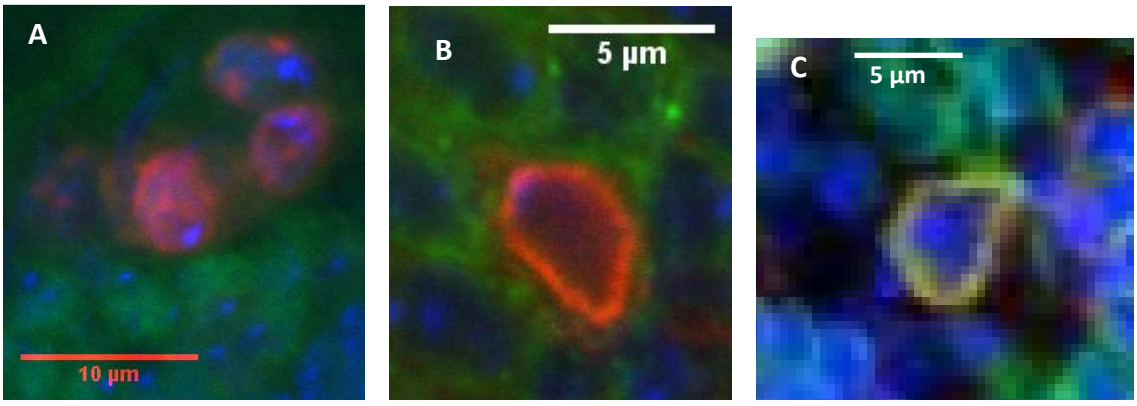



**Figure 2.9. Sub-cellular localization of NICD over-expression in glia. (A) Shows the antibody staining of Golgi with the over-expression of NICD. (B) The results of crossing the EN line to a line expressing GFP in the pattern of Calrecticulin. (C) The yellow color indicates co-localization of NICD over-expression with Lamin.**

Finally, are traditional Notch targets genes are up-regulated by the over-expression of NICD? This can be approached by crossing the EN line to a Notch transcriptional reporter gene, concatamerized Suppressor of Hairless binding sites driving a lac Z reporter gene. This would lead to over-expression of lacZ in those cells over-expressing NICD if traditional Notch target genes are activated. As can be clearly seen in Figure 2.9, this is indeed the case. Thus, over-expression of NICD in this context does lead to transcriptional up-regulation of traditional Notch target genes.

In summary, this chapter shows that Notch over-expression potentiates survival during chronic hypoxia. This NICD over-expression need only be found in a subset of glia cells in the CNS to allow survival of the whole organism. Further characterization of the Eaat1-GAL4 drivers indicates that it is expressed from embryonic stage 16 through adulthood. Subcellular localization of NICD over-expression is in or very near Lamin, a nuclear envelope structural protein. The NICD over-expression leads to up-regulation of a canonical, transcriptional Notch reporter gene. The question remains of what is the precise mechanism Notch employs to affect this phenomenon of hypoxic survival. This will be the subject of the final chapter of this dissertation.

I would like to achnwoledge my co-authors Dan Zhou, Nitin Upda, Merril Gersten, Ali Bashir, Jin Xue, Kelly A. Frazer, James W. Posakony, Shankar Subramaniam, Vineet Bafna, and Gabriel G. Haddad, who generously gave me their permission to include previously published work in Chapter 2 of this dissertation.

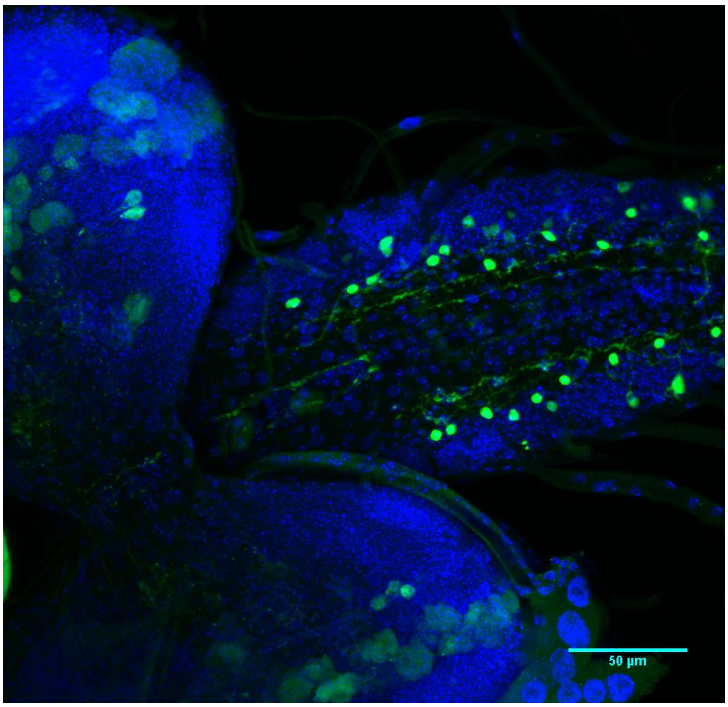



**Figure 2.10. 3rd instar larval brain of progeny of EN line crossed to Notch transcriptional reporter *gene [Su(H)]$_3$-lacZ. Hence the Eaat1-GAL4 is driving UAS-NICD which in turn should be over-expressing LacZ ; blue=DAPI, green=lacZ, z-projection of 10 slices, scale bar=50µm.***

# CHAPTER 3: MECHANISMS OF NOTCH-MEDIATED HYPOXIC SURVIVAL

## Introduction

The final chapter of this dissertation will address the question of how an increase in Notch signaling potentiates survival during hypoxia. As demonstrated in Chapter 2, the over-expression on Notch in Eaat1 glia clearly leads to survival of *Drosophila* during chronic low oxygen. Why are these particular glia cells so important to the survival of the entire organism? During development, certain glia express the high affinity glutamate transporter, Eaat1. Expression of Eaat1 has been shown to be necessary for adult survival, but not embryos or lavae (Rival et al., 2006). If Eaat1 glia are especially sensitive to hypoxic death or damage (Rival et al., 2004), then the demise or injury of Eaat1 glia could result in glutamate toxicity-mediated death of neurons (Rothstein and Kuncl, 1995), hence a non-cell autonomous effect.

Why does NICD over-expression in Eaat1 glia potentiate survival during hypoxia? Three different hypotheses on the mechanism of Notch-mediated hypoxic survival will be discussed in this chapter. First, the hypothesis of Notch up-regulation in glia cells is linked to stem cells (Gustafsson et al., 2005). This may lead to an increase in number, quality, or resistance to hypoxia of neural stem cells. For example, Notch signaling could increases the viability or integrity of stem cells, then an increase in stem cell number could allow replacement of cells injured by hypoxic insult or hypoxia-induced death. Alternatively, these stem cells may be more resistant to death or damage caused by chronic hypoxia. Secondly, the increase in Notch may lead to metabolic changes in Eaat1 glia that

increase their ability to survive during hypoxic insult. Finally, Notch may interact with other pathways such as Akt or immune pathways (NF-κB in mammals) to potentiate survival during hypoxia.

**Stem Cell Hypothesis**

During development of the larval brain, there is little increase in the number of new neurons in the first and second instar stages (Hartenstein et al., 2008). Beginning with the third instar stage, new neurons begin appearing at an exponential rate. These neurons appear at the surface of the optical lobes of the larval brain (Hartenstein et al., 2008). Careful inspection of the videos generated by confocal microscope imaging in Videos 1 and 2 in Appendix 2, shows that the majority of the glia cells over-expressing Notch are in close proximity to the developing neurons. The developing larval brain in the 3$^{rd}$ instar stage have such surface glia that could act like a stem cell niche; “the idea that glia cells act similar to a stem-cell ‘niche’ by mediating neuroblast re-entrance into the cell cycle is tempting” (Hartenstein et al., 2008).

The concept that these NICD over-expressing glia may act as a stem cell niche is supported by the stem cell niches elsewhere in *Drosophila* and humans. Succinctly defined as “locations where stem cells reside and self-renew”, a niche may be transitory, as found in the intestines of *Drosophila* (Mathur et al., 2010). Given the fact, as shown in Chapter 2, that Eaat1 expressing glia survive into adulthood, the Notch signaling may work as it does in *Drosophila* ovaries, where it establishes a permanent stem cell niche (Ward et al., 2006). In mammalian brain development, Notch also defines the niche for adult neural stem cells

(Ables et al., 2011). These glia may be transmitting a signal to neural precursor cells to allow them to divide. After amplification, these cells could then differentiate into cells (glia and/or neurons) to replace those damaged or killed due to hypoxia.

The results of determining the total volume of larval brains via confocal microcopy and staining with Grainyhead, a transcription factor that labels all post-embryonic neurons, were inconclusive. Careful examination of these suppositions in either a whole brain culture system or individually cultured cells should yield informative data. Future work could use live video imaging of dissected larval brains in normoxia and low oxygen, or the MARCM (mosaic analysis of a repressible cell marker) system of clonally labeling cells and counting the number of neurons found in each clone, comparing controls to experimental animals (Lee and Luo, 2001).

This stem cell hypothesis, with Notch signaling assisting in the formation of a niche, will afford excellent opportunities for studies in the future. If this explanation is supported by positive results, it would help explain the necessity of this subset of glia in the survival of the whole organism, especially under hypoxic stress.

**Metabolism**

Notch signaling may potentiate survival during hypoxia by modulating the metabolism of glia, allowing them to survive. Hairy, a known target of Notch in mammals, acts a metabolic switch in *Drosophila*, permitting survival during hypoxia (Zhou et al., 2008). From the Haddad lab microarray, of hypoxia-

selected larvae, pyruvate metabolism is down-regulated. In a paper entitled "Systematic Identification of Genes Promoting Hypoxic Death" (Mabon et al., 2009), pyruvate dehydrogenase (PDH) was found in an RNAi screen done in *C. elegans*. Pyruvate dehydrogenase has been linked with Notch in a large screen in *Drosophila* (Saj et al., 2010). Given these associations, PDH seems a likely candidate for interacting with the Notch signaling pathway; results from testing will be shown later in this chapter.

**NICD Crosstalk with other pathways**

Cross-talk between pathways may also be responsible for the ability of NICD over-expression to allow survival during low oxygen. The Notch signaling pathway seems to be simple and relatively straight-forward (having no second messenger); however, Notch acts directly with other diverse pathways to affect results. One such possibility is the TOR pathway through Akt (Layalle et al., 2008). Another pathway that may interact with Notch is through Relish, the mammalian NF-κB, known as part of the Imd and Toll innate immunity pathways in *Drosophila* (Lemaitre and Hoffmann, 2007).

In normal development of megakaryocytes, Notch interacts with Akt via inactivation of the Akt inhibitor PTEN (Cornejo et al., 2011). In T-cell acute lymphoblastic leukemia (T-ALL), those cancers without PTEN (which is inhibited by Notch) cannot be killed by inhibiting the Notch pathway (Gutierrez and Look, 2007). Hence, the role of Notch and Akt interaction will be tested experimentally later in this chapter.

Previous reviews have suggested that NF-kB interacts via the signaling

cell (ligand-presenting cell) in the Notch signaling pathway, by increasing the up-regulation of Jagged (Ang and Tergaonkar, 2007). However, in the experimental paradigm used in this work, the Notch signal is increased only in the recipient cell. The increase in Notch direct over-expression on NICD in glia. signaling is not due to over-expression of a Notch ligand on adjacent cells, but a direct over-expression on NICD in glia.

**Involvement of canonical Notch signaling**

The previous sections suggest that non-canonical Notch signaling (e.g. cross-talk) takes place to explain Notch-mediated hypoxic survival. However, canonical Notch signaling may also contribute to the phenomena. To test canonical Notch signaling, traditional transcriptional targets of Notch signaling should be tested. Based on availability of transgenic flies, the enhancer of split locus gene, *m-α*, will be tested later in this chapter for interactions with Notch-mediated hypoxic survival.

**Testing of NICD interaction with other pathways/genes**

Now that several candidate genes (*PDH*, *Akt*, *relish*, and *m-α*) have been identified, an experimental approach to test their interaction with NICD. The following crosses were made with the progeny assayed for survival during chronic hypoxia—see Figure 3.1. The first cross illustrates the thought that hypoxic survival is mediated through another pathway downstream of Notch signaling. From Chapter 2, we already know that Eaat1>NICD leads to better survival during hypoxia. This model adds the layer that there is another pathway with which Notch interacts to potentiates hypoxic survival. The second cross

determines the resistance to hypoxia when the gene of interest is down-regulated through RNAi in Eaat1 glia. In the second cross, an increase in survival during hypoxia suggests that the inhibition of the pathway is beneficial, and thus, cannot be tested further in this experimental paradigm. The third cross looks at the down-regulation of the gene of interest in the background of NICD over-expression. If the progeny of the third cross do not survive hypoxia as well as controls, *and* the results of the second cross also do not survive as well during hypoxia, then this suggests that Notch may be upstream of the gene of interest. Hence, this interpretation of these experiment points to a link between Notch and the gene of interest, where Notch over-regulation is upstream of the gene of interest, which in turn mediates hypoxic survival.

To ensure valid results in this experimental approach, it is necessary to balance the number of GAL4 drivers (one) with the number of UAS regulatory regions (two) within the genome of the flies to be studied. The graph below illustrates the controls necessary to determine eclosion rates with balanced numbers of drivers and regulatory regions. With more UAS regions to bind the GAL4 protein, the following graph shows that the eclosion rate will be reduced from approximately 95% to around 60%.

First, let us examine the effect of pyruvate dehydrogenase (PDH) inhibition with NICD-overexpression, as seen in Graph 3.2 below. When PDH is inhibited in Eaat1 glia, the resulting progeny survive hypoxia better than parental lines as evidenced by the red bars in Graph 3.2. Thus, PDH can be discounted as a gene acting downstream of Notch in this experimental paradigm (refer to

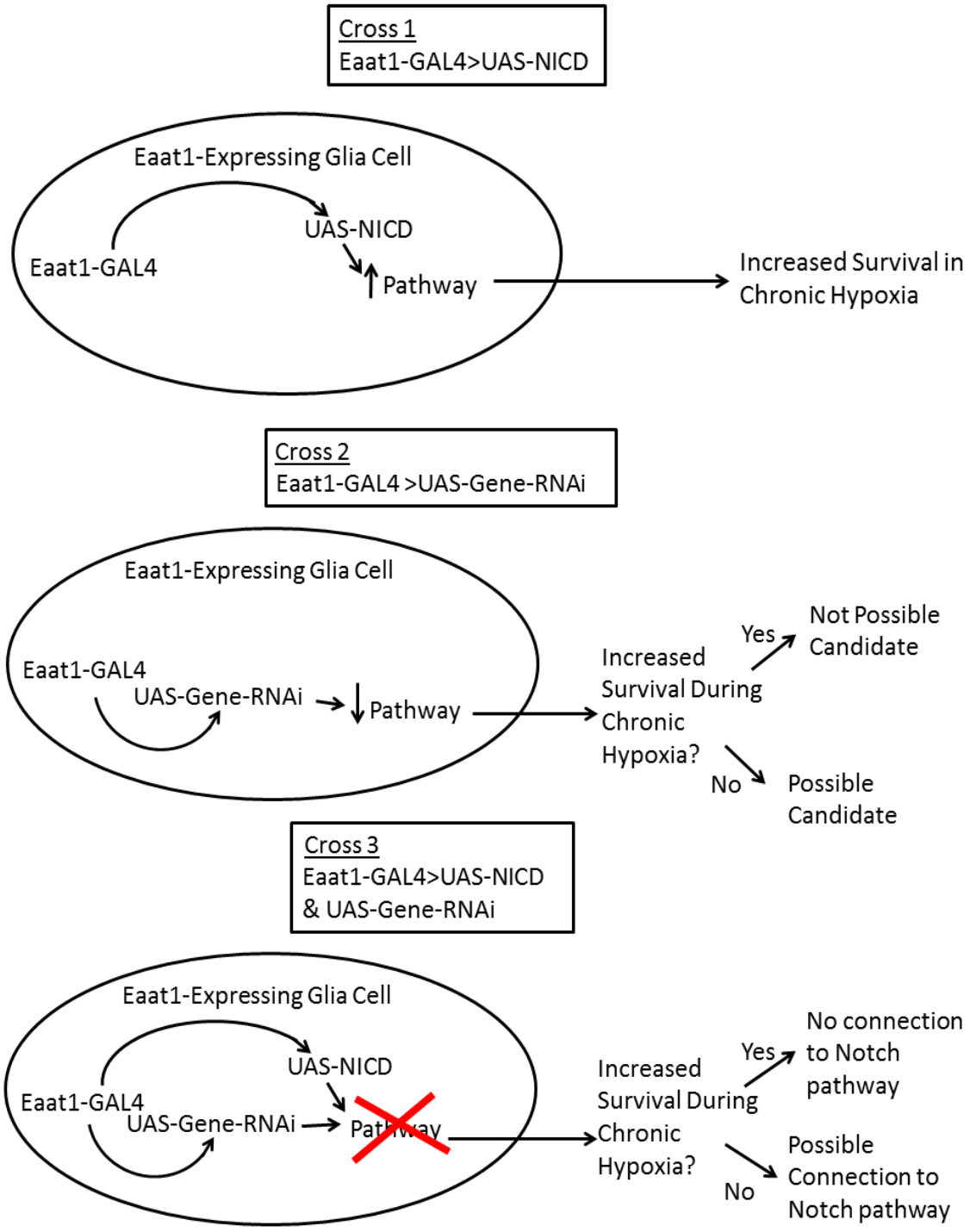
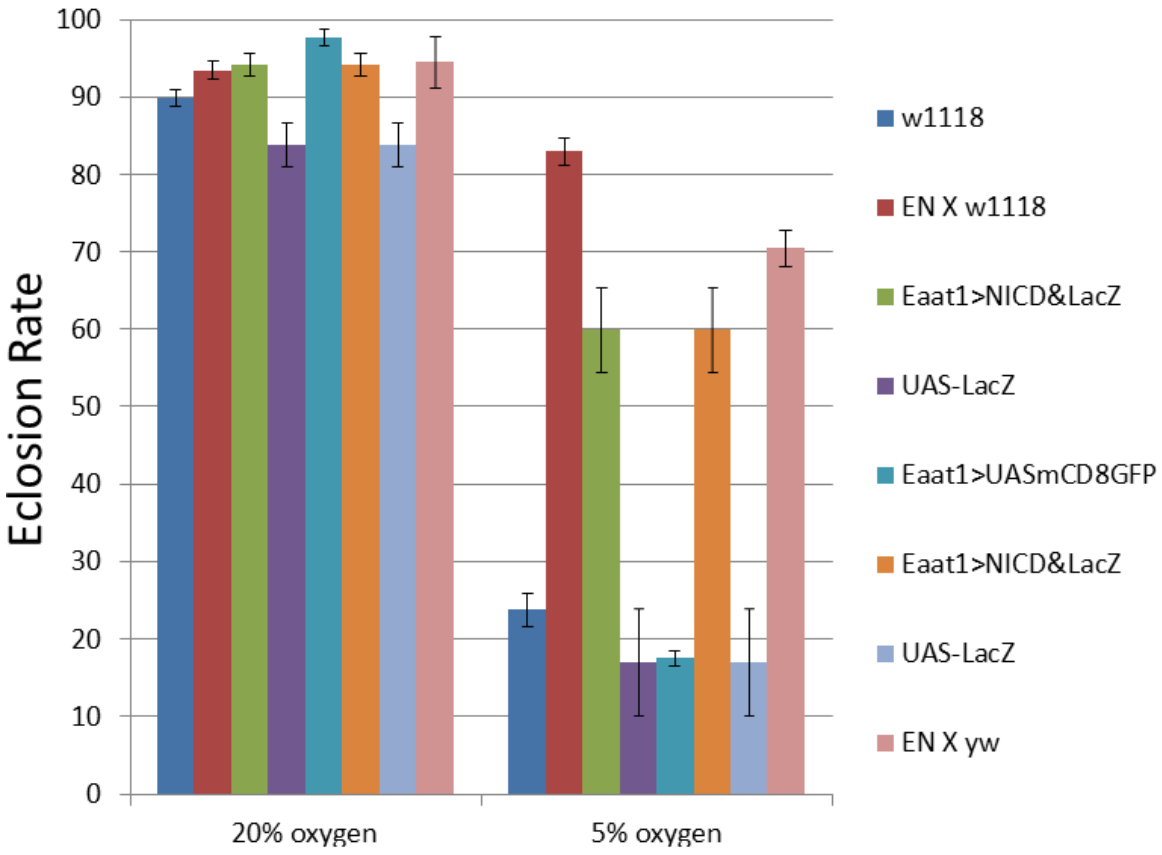



**Figure 3.1. Model of RNAi against a gene of interest in the Notch over-expression background. The first cross has already been shown to lead to increased survival of *Drosophila* during chronic low oxygen conditions. The second cross is simply the inhibition of the gene of interest with RNAi. The third cross examines the inhibition of the gene of interested in a NICD over-expression background.**

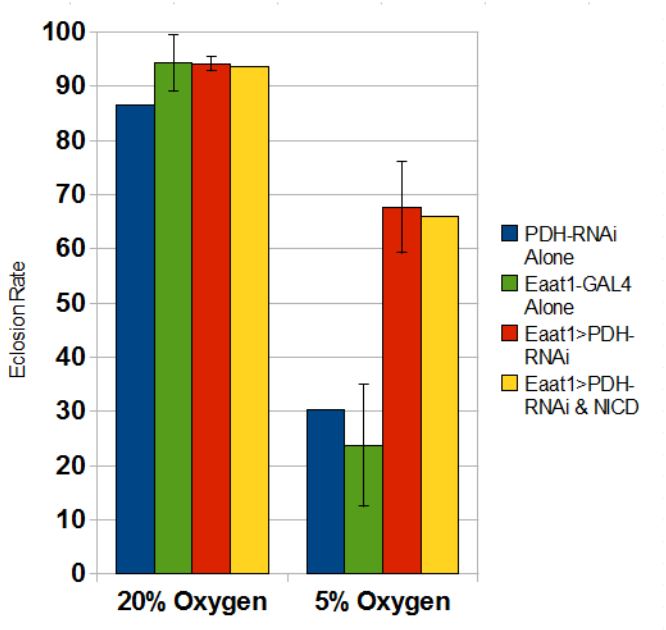
**Graph 3.1. Controls balancing the number of GAL4 and UAS construct.**




Cross 2 in Figure 3.1). Because of the results of inhibiting PDH in Eaat1 cells increases hypoxic survival, the results of inhibiting PDH with NICD over-expression are ambiguous in relation to determining an interaction with Notch. There may still indeed be an interaction--it simply cannot be determined from this data.

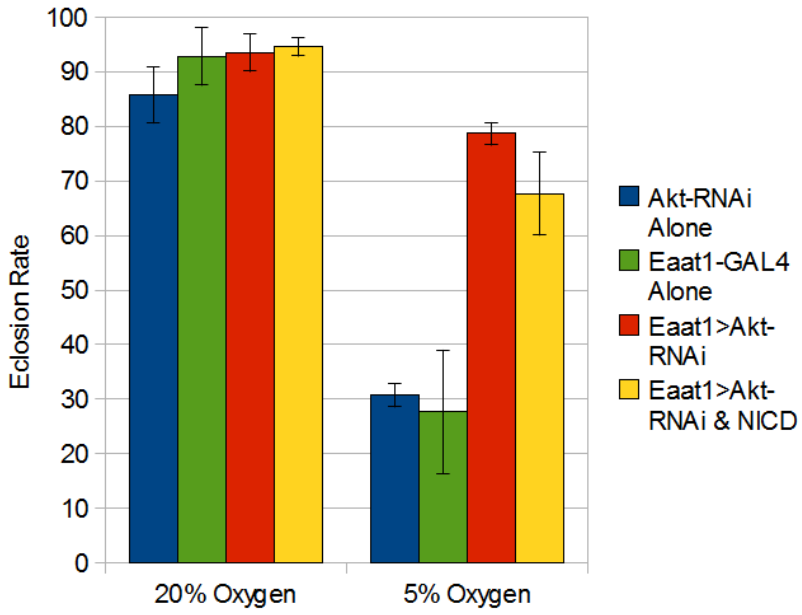
The effect of Akt is shown below in Graph 3.3. Inhibition of Akt via RNAi in Eaat1 glia leads to better survival of whole flies during hypoxia (corresponding to the 2nd cross in Figure 3.1). This suggests that Akt is not downstream of Notch, taking Graph 2.3 as a whole and recalling the interpretation of the 3rd cross in Figure 3.1

**Graph 3.2. Inhibition of PDH via RNAi with and without Notch over-expression.**




Relish acts with NICD to potentiate survival during chronic hypoxia as seen in Graph 3.4. The first two bars show the parental lines alone yielding a 30% or below eclosion rate in 5% oxygen compared to normoxia. The orange bars show that there is no improvement in hypoxic survival with the inhibition of Relish alone in Eaat1 glia. The yellow bars display the effect of inhibition Relish in the background of NICD over-expression; there is no improvement of survival with Notch over-expression if Relish expression is still repressed by RNAi. The burgundy bars demonstrate the background of flies used to generate the RNAi line (w1118) are not surviving well in hypoxia. The final light blue bars illustrate a final control where the w1118 line is crossed to the EN line, yielding one Eaat1-GAL4 driver driving one UAS-NICD construct. The final control establishes that the w1118 background has little effect on hypoxic survival when NICD is over-expressed with an 80% eclosion rate.

**Graph 3.3. Inhibition of Akt via RNAi with and without Notch over-expression.**

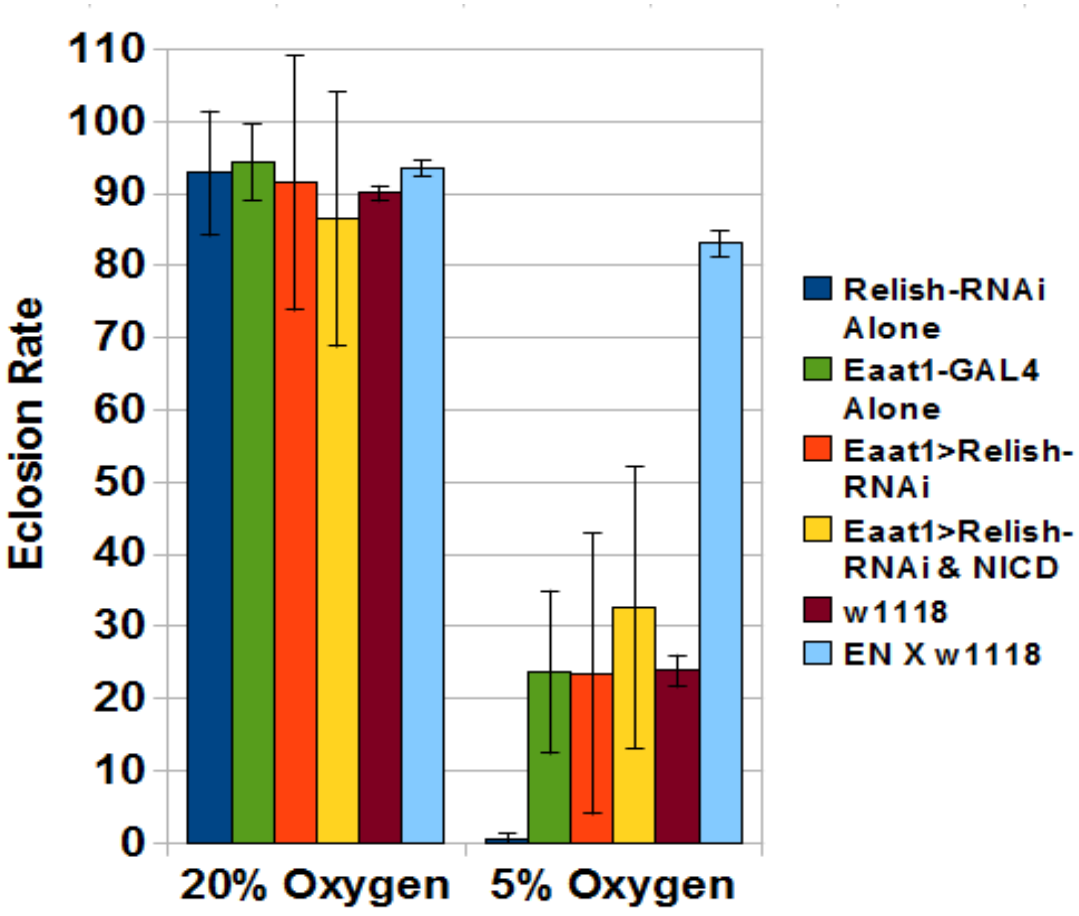



As shown by the data presented in this dissertaion, Notch signaling leads to an increase in hypoxic survival via Relish. This is illustrated in Figure 3.2 below. Future work will address the question of whether this results in an increase in target genes of Relish (anit-microbial peptides, other survival signals, etc.). It is enticing to speculate that there may be a direct interaction between Notch and Relish. Published work indicated that there is a direct interaction between Notch and NF-κB during T-cell activation. This results in a sustained output of genes induced by NF-κB (Shin et al., 2006). The mechanism for this is via the retention of NF-κB in the nucleus. NICD could bind to Relish and prevent its nuclear export. In turn, because Relish in retained in the nucleus, there is a sustained expression of downstream target genes of Relish, leading the survival during chronic hypoxia.

This data contributes to the idea that over-expression of NICD leads to up-

regulation of immune pathways via Relish (p65 in mammals. In *Drosophila*, downstream transcriptional targets of Relish include anti-microbial peptides (AMP) (Dushay et al., 1996). These AMP's have been shown to increase resistance of flies to oxidative stress (Zhao et al., 2011). The mechanism for this increased resistance may be due to ability of the charged peptides to bind reactive oxygen species (ROS) removing them from the cytocylic milieu. Alternatively, AMPs form a pore in bacteria, yeast, and fungi to act as part of the innate immunity in Drosophila (Bulet et al., 1999). This pore may act as a multi-drug resistant gene, which forms an ion channel to export

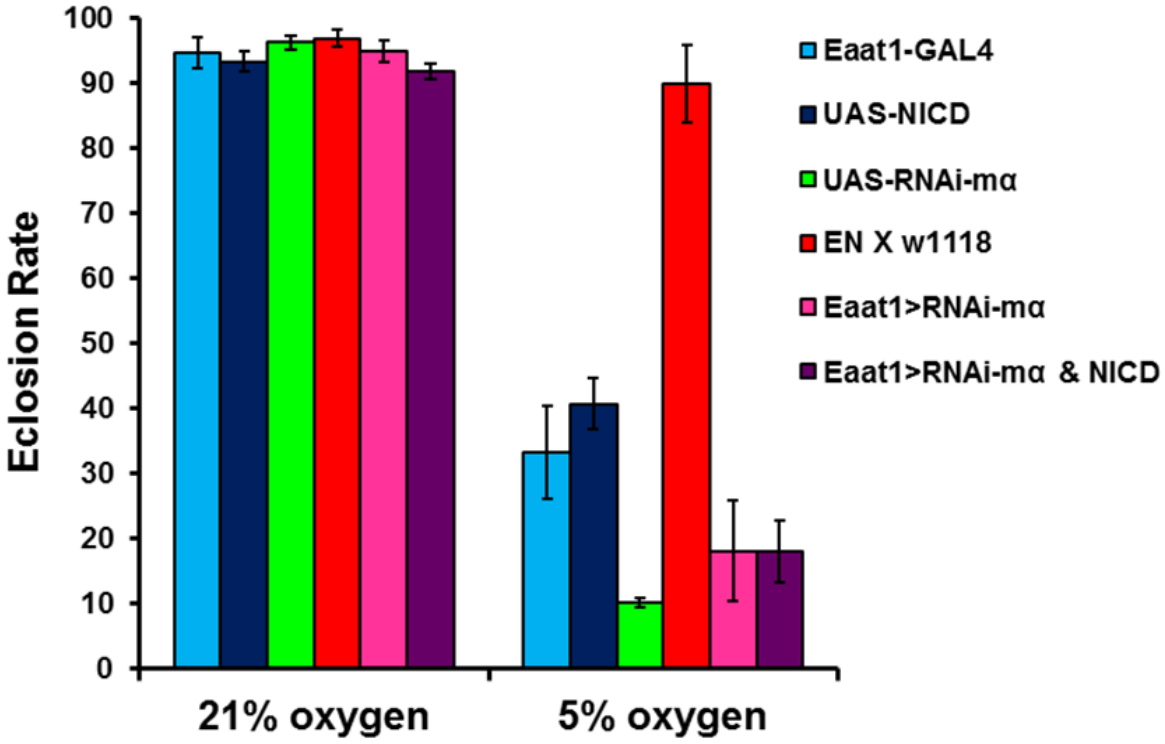
**Graph 3.4. Relish RNAi in NICD over-expression background.**



unkown targets, such as ROS or toxic metalbolites, from a cell during hypoxia (Huang and Haddad, 2007). The effect of increased immune signaling in neural cells may facilitate neural stem cell development (Ang and Tergaonkar, 2007).

The following graph (Graph 3.5) illustrates that the inhibition of m-α decreases survival during chronic hypoxia compared to controls. Inhibition of m-α in Eaat1 glia with either NICD over-expression, or lack thereof, has any effect on whole organism survival during chronic hypoxia. Interpreting this data with the explanation in Figure 3.1 points to the conclusion that m-α also acts to explain Notch mediated hypoxic survival. This suggests that canonical Notch signaling is also a mechanism that potentiates survival during chronic hypoxia.

**Graph 3.5. Inhibition of m-α in Eaat1 glia decreases survival during chronic hypoxia**




**Model of NICD and Relish Interactions**

As shown by the data presented in this dissertation, Notch signaling leads to an increase in hypoxic survival via Relish. This is illustrated in Figure 3.2 below. Future work will address the question of whether this results in an increase in target genes of Relish (anit-microbial peptides, other survival signals, etc).

It is enticing to speculate that there is a direct interaction between Notch and Relish in *Drosophia* glia. Published work indicates that there is a direct interaction between Notch and Relish in the nucleus of cells during T-cell activation. This results in a sustained output of genes induced by NF-κB (Shin et al., 2006). The mechanism for this is via the rentention of NF-κB in the nucleus. NICD could bind to Relish and thus prevents its nuclear export. In turn, because Relish is retained in the nucleus, there is a sustained expression of downstream targets of Relish, leading to the potentiation of survival during chronic hypoxia. These ideas can be tested in the future with immunoprecipitations, confocal microscopy of stained larval brains, qPCR, whole *Drosophila* brain culture, and tissue culture.

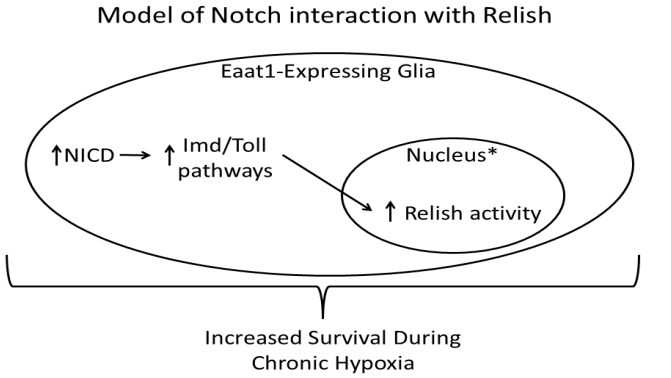


**Figure 3.2. Model of NICD interaction with Relish**

## Conclusion

This dissertation and other published work establish solid evidence that Notch signaling is linked to hypoxic survival. Notch signaling is extremely context dependent, both temporally and spatially, as well as dependent on levels of expression. The particular experimental paradigm used in these studies is glia over-expressing NICD during embryogeneis and into adulthood. This over-expression leads to an increase in survival of the whole organism during chronic hypoxia. The data suggests that both traditional Notch signaling pathways (e.g. m-α) and NICD interactions with immune pathways via Relish assist in this phenomena. Given the intricacies of Notch signaling, this is a fertile area for more research. This dissertation had laid the ground work for future studies into the precise nature of Notch-mediated hypoxic survival.

## APPENDIX 1: METHODS AND MATERIALS

**Fly culture.** *Drosophila* cultures were maintained on standard yeasted cornmeal molasses media made by the UCSD staff. Cultures were changed every 3 to 4 weeks to maintain stocks and maintained in the Haddad lab fly room at approximately 22 degrees Centigrade. Virgin females were selected based on the presence of meconium in the gut and aged 3 to 5 days before crosses to males. The vials in which the females were aged were saved for one to two weeks to ensure no eggs hatched. Unless otherwise noted, 10 virgin females were crossed to 5 males aged 2 to 7 days.

**Fly crosses to generate data for eclosion rates and adult post-eclosion survival.** Three to five day old UAS-NICD virgin females were crossed to GAL4 males and allowed to mate and lay eggs for 48 hours in ambient conditions. The adults were then placed in another vial (for controls), allowed to continue mating and laying eggs for 48 more hours, then the parents were discarded and control vials were allowed to develop in normal oxygen conditions. Meanwhile, the experimental vials were placed in a 5% oxygen chamber and allowed to develop. Once eclosed, adults were moved to a new vial once a day, with experimental flies continuing in 5% oxygen and control flies continuing in room air. Once all adults were eclosed and transferred, the original vial was used to count the number of empty pupal cases and the number of not-empty pupal cases to determine the eclosion rate. The adult post-eclosion rate was determined by counting the total number of surviving adults.

**Immunohistochemical staining.** Larval and adult brains were dissected

in 1X PBS (phosphate buffered saline) and immediately fixed in 4% paraformaldehyde in 1X PBS for 30 minutes with gentle mechanical swirling utilized throughout the following steps. All steps were carried out at room temperaure unless otherwise noted. PBS with 0.3% Triton X-100 was employed to permeabilize the membranes of the brains with 3, 15 minute washes. Blocking occurred in in 7% normal goat serum in 1X PBS plus 0.3% TritonX-100 for 30 minutes. Primary antibody [NICD(mouse), undiluted filtered supernatant from hybirdoma cells, repo (rabbit) 1:100 diluted into NICD supernatant, elav (rat) 1:50 diluted into NICD supernatant] incubated with the brains overnight at 4 degrees C for 1 hour at room temperature. This was followed with 3, 15 minute washes of 1X PBS plus 0.3% TritonX-100. Secondary antibobdy (Invitrogen Alexa-Fluorophore 1:250) was diluted into 1X PBS plus 0.3% TritonX-100 with 7% normal goat serum with a 1.5 to 2 hour incubation in the dark. This was followed with 3, 15 minute washes of 1X PBS plus 0.3% TritonX-100. Finally the brains were mounted (using a dissecting microscope) on pre-cleaned glass microscope slides in Invitrogen ProLong AntiFade Gold and a glass coverslip was sealed in place by a “berm” of clear nail polish. The slides were allowed to cure in the dark at room temperature overnight, and then refrigerated in the dark, both before and after imaging. The protocol for fixing embryos utilizes the standard heptane protocol to preserve the GFP epitope. After the embryos were fixed, the protocol above from the permeabilization step.

**Imaging.** Confocal images were taken on an FV1000 Olympus Microscope in the UCSD Neuosciencel Microscopy Core with 400 to 600X

magnification. Sections were imaged at 2 micron intervals. Data was processed with ImageJ from NIH.

**Statistics.** Unpaired t-test and standard error of the mean (SEM) calculated in Excel 2007 by Microsoft with the exception of Graph 2.2 and 2.3 in which the SEM was calculated in GraphPad Prism version 4.03 for PC, GraphPad Software, San Diego California USA, www.graphpad.com.

# APPENDIX 2: VIDEOS

**Video 1. 3rd instar larval brain Eaat1>NICD; red=repo, blue=Elav, green=NICD; scale bar is 50µm.**

Eaat1-NICD.avi

**Video 2. 3rd instar larval brain 17A>NICD; red=repo, blue=Elav, green=NICD; scale bar is 50µm.**

17A-NICD.avi

REFERENCES

**Ables, J. L., Breunig, J. J., Eisch, A. J. and Rakic, P.** (2011). Not(ch) just development: Notch signalling in the adult brain. *Nature Reviews Neuroscience* **12**, 269-283.

**Androutsellis-Theotokis, A., Leker, R. R., Soldner, F., Hoeppner, D. J., Ravin, R., Poser, S. W., Rueger, M. A., Bae, S. K., Kittappa, R. and McKay, R. D. G.** (2006). Notch signalling regulates stem cell numbers in vitro and in vivo. *Nature* **442**, 823-826.

**Ang, H. L. and Tergaonkar, V.** (2007). Notch and NF kappa B signaling pathways: do they collaborate in normal vertebrate brain development and function? *Bioessays* **29**, 1039-1047.

**Bulet, P., Hetru, C., Dimarcq, J. L. and Hoffmann, D.** (1999). Antimicrobial peptides in insects; structure and function. *Developmental and Comparative Immunology* **23**, 329-344.

**Cornejo, M. G., Mabialah, V., Sykes, S. M., Khandan, T., Lo Celso, C., Lopez, C., Rivera-Munoz, P., Rameau, P., Tothova, Z., Aster, J. C. et al.** (2011). Crosstalk between Notch and AKT signaling during murine megakaryocyte lineage specification. *Blood*.

**delaPompa, J., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. et al.** (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139-1148.

**Dushay, M. S., Asling, B. and Hultmark, D.** (1996). Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 10343-10347.

**Fortini, M. E., Hu, Y. and Seidner, G. A.** (2004). Exploring the biology of gamma-secretase in Drosophila. *Neurobiology of Aging* **25**, S46-S46.

**Gustafsson, M. V., Zheng, X. W., Pereira, T., Gradin, K., Jin, S. B., Lundkvist, J., Ruas, J. L., Poellinger, L., Lendahl, U. and Bondesson, M.**

(2005). Hypoxia requires Notch signaling to maintain the undifferentiated cell state. *Developmental Cell* **9**, 617-628.

**Gutierrez, A. and Look, A. T.** (2007). NOTCH and PI3K-AKT pathways intertwined. *Cancer Cell* **12**, 411-3.

**Haddad, G. G. and Ma, E.** (2001). Neuronal tolerance to O2 deprivation in drosophila: novel approaches using genetic models. *The Neuroscientist* **7**, 538-50.

**Hartenstein, V., Spindler, S., Pereanu, W. and Fung, S.** (2008). The development of the Drosophila larval brain. In *Brain Development in Drosophila Melanograster*, vol. 628, pp. 1-31.

**Huang, H. and Haddad, G. G.** (2007). Drosophila dMRP4 regulates responsiveness to O2 deprivation and development under hypoxia. *Physiol Genomics* **29**, 260-6.

**Kopan, R. and Goate, A.** (2002). Aph-2/nicastrin: An essential component of gamma-secretase and regulator of notch signaling and presenilin localization. *Neuron* **33**, 321-324.

**Layalle, S., Arquier, N. and Leopold, P.** (2008). The TOR Pathway Couples Nutrition and Developmental Timing in Drosophila. *Developmental Cell* **15**, 568-577.

**Lee, T. M. and Luo, L. Q.** (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development (vol 24, pg 251, 2001). *Trends in Neurosciences* **24**, 385-385.

**Lemaitre, B. and Hoffmann, J.** (2007). The host defense of Drosophila melanogaster. *Annual Review of Immunology* **25**, 697-743.

**Lloyd-Jones, D., Adams, R. J., Brown, T. M., Carnethon, M., Dai, S., De Simone, G., Ferguson, T. B., Ford, E., Furie, K., Gillespie, C. et al.** (2010). Executive Summary: Heart Disease and Stroke Statistics-2010 Update A Report From the American Heart Association. *Circulation* **121**, 948-954.

**Mabon, M. E., Mao, X. R., Jiao, Y., Scott, B. A. and Crowder, C. M.** (2009). Systematic Identification of Gene Activities Promoting Hypoxic Death. *Genetics* **181**, 483-496.

**Mathur, D., Bost, A., Driver, I. and Ohlstein, B.** (2010). A Transient Niche Regulates the Specification of Drosophila Intestinal Stem Cells. *Science* **327**, 210-213.

**Morgan, T. H.** (1917). The Theory of the Gene. *The American Naturalist* **51**, 513-544.

**Mourikis, P., Lake, R. J., Firnhaber, C. B. and DeDecker, B. S.** (2010). Modifiers of notch transcriptional activity identified by genome-wide RNAi. *BMC Developmental Biology* **10**.

**Mummery-Widmer, J. L., Yamazaki, M., Stoeger, T., Novatchkova, M., Bhalerao, S., Chen, D., Dietzl, G., Dickson, B. J. and Knoblich, J. A.** (2009). Genome-wide analysis of Notch signalling in Drosophila by transgenic RNAi. *Nature* **458**, 987-U59.

**Okajima, T., Matsuura, A. and Matsuda, T.** (2008a). Biological functions of glycosyltransferase genes involved in O-fucose glycan synthesis. *Journal of Biochemistry* **144**, 1-6.

**Okajima, T., Reddy, B., Matsuda, T. and Irvine, K. D.** (2008b). Contributions of chaperone and glycosyltransferase activities of O-fucosyltransferase 1 to Notch signaling. *BMC Biology* **6**.

**Pasteur, L., Faulkner, F. and Robb, D. C.** (1879). Studies on fermentation: The diseases of beer, their causes, and the means of preventing them: Macmillan & co.

**Phelps, C. B. and Brand, A. H.** (1998). Ectopic gene expression in Drosophila using GAL4 system. *Methods* **14**, 367-379.

**Prasad, S. M., Czepiel, M., Cetinkaya, C., Smigielska, K., Weli, S. C., Lysdahl, H., Gabrielsen, A., Petersen, K., Ehlers, N., Fink, T. et al.** (2009). Continuous hypoxic culturing maintains activation of Notch and allows long-term propagation of human embryonic stem cells without spontaneous differentiation. *Cell Proliferation* **42**, 63-74.

**Quinones-Coello, A. T., Petrella, L. N., Ayers, K., Melillo, A., Mazzalupo, S., Hudson, A. M., Wang, S., Castiblanco, C., Buszczak, M., Hoskins, R. A. et al.** (2007). Exploring strategies for protein trapping in Drosophila. *Genetics* **175**, 1089-1104.

**Rival, T., Soustelle, L., Cattaert, D., Strambi, C., Iche, M. and Birman, S.** (2006). Physiological requirement for the glutamate transporter dEAAT1 at the adult Drosophila neuromuscular junction. *Journal of Neurobiology* **66**, 1061-1074.

**Rival, T., Soustelle, L., Strambi, C., Besson, M. T., Iche, M. and Birman, S.** (2004). Decreasing glutamate buffering capacity triggers oxidative stress and neuropil degeneration in the Drosophila brain. *Curr Biol* **14**, 599-605.

**Rothstein, J. D. and Kuncl, R. W.** (1995). NEUROPROTECTIVE STRATEGIES IN A MODEL OF CHRONIC GLUTAMATE-MEDIATED MOTOR-NEURON TOXICITY. *Journal of Neurochemistry* **65**, 643-651.

**Saj, A., Arziman, Z., Stempfle, D., van Belle, W., Sauder, U., Horn, T., Durrenberger, M., Paro, R., Boutros, M. and Merdes, G.** (2010). A Combined Ex Vivo and In Vivo RNAi Screen for Notch Regulators in Drosophila Reveals an Extensive Notch Interaction Network. *Developmental Cell* **18**, 862-876.

**Sakata, T., Sakaguchi, H., Tsuda, L., Higashitani, A., Aigaki, T., Matsuno, K. and Hayashi, S.** (2004). Drosophila Nedd4 regulates endocytosis of notch and suppresses its ligand-independent activation. *Current Biology* **14**, 2228-2236.

**Schweisguth, F.** (2004). Regulation of notch signaling activity. *Current Biology* **14**, R129-R138.

**Shah, S., Lee, S. F., Tabuchi, K., Hao, Y. H., Yu, C., LaPlant, Q., Ball, H., Dann, C. E., 3rd, Sudhof, T. and Yu, G.** (2005). Nicastrin functions as a gamma-secretase-substrate receptor. *Cell* **122**, 435-47.

**Shin, H. M., Minter, L. M., Cho, O. H., Gottipati, S., Fauq, A. H., Golde, T. E., Sonenshein, G. E. and Osborne, B. A.** (2006). Notch1 augments NF-kappa B activity by facilitating its nuclear retention. *EMBO Journal* **25**, 129-138.

**Sriuranpong, V., Borges, M. W., Strock, C. L., Nakakura, E. K., Watkins, D. N., Blaumueller, C. M., Nelkin, B. D. and Ball, D. W.** (2002). Notch signaling

induces rapid degradation of achaete-scute homolog 1. *Mol Cell Biol* **22**, 3129-39.

**Stifani, S., Blaumueller, C. M., Redhead, N. J., Hill, R. E. and Artavanis-Tsakonas, S.** (1992). Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins. *Nat Genet* **2**, 343.

**Wang, R., Zhang, Y. W., Zhang, X., Liu, R., Zhang, X., Hong, S., Xia, K., Xia, J., Zhang, Z. and Xu, H.** (2006). Transcriptional regulation of APH-1A and increased gamma-secretase cleavage of APP and Notch by HIF-1 and hypoxia. *Faseb J* **20**, 1275-7.

**Ward, E. J., Shcherbata, H. R., Reynolds, S. H., Fischer, K. A., Hatfield, S. D. and Ruohola-Baker, H.** (2006). Stem cells signal to the niche through the notch pathway in the Drosophila ovary. *Current Biology* **16**, 2352-2358.

**Werner, T.** (2008). Bioinformatics applications for pathway analysis of microarray data. *Current Opinion in Biotechnology* **19**, 50-54.

**Zhao, H. W., Zhou, D. and Haddad, G. G.** (2011). Antimicrobial Peptides Increase Tolerance to Oxidant Stress in Drosophila melanogaster. *Journal of Biological Chemistry* **286**, 6211-6218.

**Zhou, D., Visk, D. W. and Haddad, G. G.** (2009). Drosophila, a golden bug, for the dissection of the genetic basis of tolerance and susceptibility to hypoxia. *Pediatr Res* **66**, 239-47.

**Zhou, D., Xue, J., Chen, J., Morcillo, P., Lambert, J. D., White, K. P. and Haddad, G. G.** (2007). Experimental selection for Drosophila survival in extremely low O(2) environment. *PLoS One* **2**, e490.

**Zhou, D., Xue, J., Lai, J. C. K., Schork, N. J., White, K. P. and Haddad, G. G.** (2008). Mechanisms Underlying Hypoxia Tolerance in Drosophila melanogaster: hairy as a Metabolic Switch. *PLoS Genetics* **4**.