IMPL2 REPRESSES INSULIN SIGNALING IN RESPONSE TO HYPOXIA

by

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A DISSERTATION

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

June 2011

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Title: ImpL2 Represses Insulin Signaling in Response to Hypoxia

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DISSERTATION ABSTRACT

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Doctor of Philosophy

Department of Biology

June 2011

Title: ImpL2 Represses Insulin Signaling in Response to Hypoxia

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Correct regulation of insulin/insulin-like growth factor signaling (IIS) is essential for proper development and growth. More recently, proper regulation of IIS has been shown to be important for adaptation and survival under stressful conditions. Despite the importance of IIS, the mechanism underlying IIS regulation under various environmental stresses remains to be elucidated. One mechanism of regulating IIS involves the binding of insulin and insulin-like growth factors by insulin-like growth factor binding proteins (IGFBPs), which prevent the factors from interacting with the insulin receptor (InR). The only identified IGFBP in *Drosophila* to date is imaginal morphogenesis protein late 2 (Imp-L2), which was previously implicated in the regulation of IIS during starvation. Here, we investigate whether Imp-L2 is required to regulate IIS under low oxygen stress (hypoxia).

The ability to tolerate hypoxia requires cellular adaptations that decrease the need for oxygen and increase the supply of it. In a wide variety of organisms many of these adaptations are either directly or indirectly regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1). Our results reveal a regulatory link between HIF-1, Imp-L2, and IIS during hypoxia. We demonstrate that Imp-L2 transcript abundance is increased during

hypoxia in a HIF-1 dependent manner resulting in inhibition of IIS and increased hypoxia tolerance.

This dissertation includes unpublished co-authored material.

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Allee, J. P., Tran, K. D., Turnbull, D. W., Doe, C. Q., Johnson, E. A. *Drosophila* Insulin Pathway Activity is Repressed During Hypoxia by Imp-L2 in a HIF-1 Dependent Manner. (Manuscript under review)

Truman, J. W., Hiruma, K., Allee, J. P., Macwhinnie, S. G., Champlin, D. T. and Riddiford, L. M. Juvenile hormone is required to couple imaginal disc formation with nutrition in insects. (2006) *Science* **312**, 1385-1388

Allee, J. P., Pelletier, C. L., Fergusson, E. K. and Champlin, D. T. Early events in adult eye development of the moth, Manduca sexta. (2006) *J Insect Physiol* **52**, 450-460

MacWhinnie, S. G., Allee, J. P., Nelson, C. A., Riddiford, L. M., Truman, J. W. and Champlin, D. T. The role of nutrition in creation of the eye imaginal disc and initiation of metamorphosis in Manduca sexta. (2005) *Dev Biol* **285**, 285-297

ACKNOWLEDGMENTS

I wish to express my utmost gratitude to my wife Jenn for her steadfast love and unending encouragement all these years. I wish to thank my advisor, Dr. Eric Johnson, for allowing me the freedom to explore the biological questions I found interesting and to manage my time in a way that worked best for me. I also wish to thank my undergraduate research advisor and good friend, Dr. Dave Champlin, for truly mentoring me in science and inspiring me to pursue a PhD.

I would like to give thanks to J. T. Neal for technical assistance with Relative qPCR data analysis and to Ryan Andersen for technical assistance with confocal microscopy.

The project described in Chapter II was supported by grants R21HG003834 and R21HG006036 from the National Human Genome Research Institute (E.A.J.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Human Genome Research Institute or the National Institutes of Health.

I would like to dedicate this dissertation to God who has equipped me to do good works.

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CHAPTER I

INTRODUCTION TO INSULIN/INSULIN-LIKE GROWTH FACTOR SIGNALLING AND HYPOXIC GENE REGULATION

Background to Insulin Signaling

Insulin and insulin like growth factors (IGFs), along with their respective receptors and the insulin-like growth factor binding proteins (IGFBPs) all fall into the insulin superfamily of growth-promoting peptides and comprise the core components of the IGF system (1). Insulin/insulin-like growth factor signaling (IIS) is responsible for the regulation of cell survival, cell cycle kinetics, proliferation, body size, metabolism, fertility, and longevity in both vertebrates and invertebrates (2-4). IIS activity is regulated through both intracellular and extracellular signaling pathways, which includes the production and release of ligands and receptors, and through the binding activity of IGFBPs (2,3). It has been proposed that an ancient insulin-like gene with predominantly mitogenic properties was the common ancestor to insulin and IGFs I and II found in vertebrates as well as the insulin like peptides (ILPs) found in invertebrates as they all exhibit a high degree of homology (5).

In vertebrates, the insulin receptor (IR), IGF-I receptor (IGF-IR) and IGF-II receptor (IGF-IIR) are preferentially but not exclusively bound by their respective ligands as IGF-II also binds the IR and the IGF-IR. Additionally, hybrid IGF/insulin receptors have been identified that increase the complexity of IGF system by varying the affinities of the receptors for the different ligands (6). Six IGFPBs have been identified in

vertebrates that bind the IGFs with high affinity and control their availability for receptor binding and as well as increase their half-lives in circulation and extracellular fluids. While insulin occurs in free form, the IGFs are all found in ternary complexes with IGFBPs and acid-labile subunit (ALS) in a 1:1:1 molar ratio (2,3,6). The binding of the IR or IGF-I by their respective ligands stimulates tyrosine-kinase pathways that initiate intracellular signaling cascades that are similar at their proximal ends. Divergence of the signaling pathways results in insulin primarily regulating metabolism and the IGFs primarily regulating mitogenic events. The IGF-IIR is though to primarily function in the removal of IGF-II from extracellular fluid (1,6).

InR is the fruit fly (*Drosophila melanogaster)* homologue of the IR and IGF-IR in vertebrates with structural similarities to both (7). Additionally, seven insulin-like peptides have been identified in *Drosophila* (Dilp 1-7) that are homologous to vertebrate insulin and IGF-1; however, the first IGFBP homologue was only identified recently (8). Imaginal morphogenesis protein late 2 (Imp-L2) has been identified as an insulin-like growth factor binding protein (IGFBP) and putative homolog of vertebrate IGFBP-7 (8,9). The superfamily of IGFBPs includes the six high-affinity IGFBPs and several low-affinity binding proteins referred to as IGFBP related proteins (IGFBP-rP), which includes IGFBP-7 (10). One aspect of IIS that is not well understood is the regulation of IGFBP expression and activity under varying conditions. The discovery of an invertebrate IGFBP homologue provides the opportunity to further dissect IIS in a simpler model system.

A better understanding of IIS is essential as malfunctions of this system are involved in many human diseases. In addition to the role of insulin in the metabolic

disorder diabetes mellitus, malfunction of the IGF system is strongly associated with a variety of human cancers. Increased transcription of one or more of the genes encoding IGF-I, IGF-II, IGF-IR and IR-A are observed in malignant tumors in various central nervous system cancers as well as prostate, breast, lung, colon, rectum, pancreas, liver and ovarian cancer. On a broader scale, an increased risk of all-cause, cardiovascular and cancer mortality was observed in men with low IGF-I serum level concentrations and an increased risk of all-cause mortality was observed in men and women with low serum level concentrations of IGFBP-3 (1).

Background to Hypoxia

All eukaryotic organisms require molecular oxygen (O_2) for cellular respiration. Aerobic respiration takes place in mitochondria where oxygen is the final electron acceptor during oxidative phosphorylation to generate adenosine triphosphate (ATP). In vertebrates, oxygen diffuses through alveoli in the lungs where is it delivered to hemoglobin for transport throughout the vascular system to the tissues where it is needed. *Drosophila* use a system of spiracles and trachea to deliver oxygen to tissues. Since oxygen is essential for the growth and survival of all obligate aerobes, low oxygen stress (hypoxia) is a key concern. A common observation is that hypoxia results in the suppression of somatic growth in humans at high altitude or with congenital heart disease or in infants with chronic lung disease. Similarly, *Drosophila* raised under hypoxia are smaller and take longer to develop (11).

The ability to tolerate both acute and chronic hypoxia requires cellular adaptations that are mediated by hypoxia responsive transcription factors. In most, if not all, aerobic

organisms, the master regulator of the hypoxic response is the transcription factor hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimeric protein consisting of an α-subunit and a β-subunit. While both subunits are constitutively expressed, the α-subunit is rapidly degraded under normoxic conditions. When oxygen is scarce, the α-subunit accumulates in the cytoplasm, binds to the β-subunit to form active HIF-1, and translocates to the nucleus to regulate essential target genes (11-13). The transcriptional regulation of target genes allows cells to change their physiology in ways that allow them to better cope with hypoxia.

Bridge to Chapter II

In the previous chapter I described the fundamentals of insulin/IGF signaling in both vertebrates and invertebrates. Additionally, I described regulation of the transcriptional response to hypoxia. In Chapter II I report data form a paper currently under review for publication, of which I was the first author, which examined the function and regulation of an IGFBP during hypoxia in *Drosophila melanogaster.* The co-authors are Khoa D. Tran, Douglas W. Turnbull and Eric A. Johnson.

CHAPTER II

DROSOPHILA INSULIN PATHWAY ACTIVITY IS REPRESSED DURING HYPOXIA BY IMPL2 IN A HIF-1 DEPENDENT MANNER

Reproduced from J. Paul Allee, Khoa D. Tran, Douglas W. Turnbull, and Eric A. Johnson

Submitted *to Journal of Biological Chemistry* May 6, 2011

Khoa Tran contributed to this work by conducting antibody stains in embryos and larvae and by taking microscope images. Douglas Turnbull contributed by exposing wild-type and HIF-1α homozygous mutant adult flies to hypoxia and isolating their RNA. I conducted the remainder of the experiments and was responsible for the experimental design as well as the data analysis and writing.

Insulin/insulin-like growth factor signaling (IIS) is responsible for the regulation of cell survival, the cell cycle, proliferation, body size, metabolism, fertility, and longevity in both vertebrates and invertebrates (2-4). IIS activity is regulated through both intracellular and extracellular signaling pathways, which includes the production and release of ligands and receptors, and through the binding activity of insulin-like growth factor binding proteins (IGFBPs) (2,3). One aspect of IIS regulation that is not well understood is the control of IGFBP expression and activity under varying conditions. IGFBPs regulate the availability and activity of insulin-like growth factors (IGFs) by binding them with high affinity, thus preventing them from interacting with the

insulin receptor (InR) (2). Additionally, the formation of ternary complexes between IGFs, acid labile subunit (ALS) and IGFBPs prolongs the half-lives of IGFs and protects them from degradation (2,3). While seven insulin-like peptides have been identified in Drosophila (Dilp 1-7) that are homologous to vertebrate insulin and IGF-1, the first IGFBP homologue was only identified recently (8). Imaginal morphogenesis protein late 2 (ImpL2) has been identified as an IGFBP and putative homolog of vertebrate IGFBP-7 (8,9). This provides an opportunity to study regulation of IGFBP production and activity using the Drosophila model.

ImpL2 was initially identified as one of six ecdysone inducible genes expressed during imaginal disc morphogenesis (14). Drosophila ImpL2 was later reported to be a secreted member of the immunoglobulin superfamily implicated in neural and ectodermal development based on patterns of mRNA expression (15). In 2000 a Drosophila ImpL2 homologue was identified that could bind insulin and related peptides in the insect Spodoptera frugiperda, and later Drosophila ImpL2 was shown to bind to and antagonize Drosophila insulin like peptide 2 (Dilp2) and inhibit IIS (8,16,17). Although ImpL2 is not essential in standard conditions, homozygous mutants are larger than wild-type. During adverse nutritional conditions ImpL2 expression increases in the fat body, and homozygous mutants have much higher mortality rates (8). These results provide a strong argument that ImpL2 is an IGFBP that not only plays a role in regulating growth during development, but also is also required for regulating the activity of IIS during starvation.

The ability to tolerate hypoxia requires cellular adaptations that decrease the need for oxygen and increase the supply of it. In a wide variety of organisms, many of these adaptations are either directly or indirectly regulated by the transcription factor Hypoxia-

inducible factor-1 (HIF-1) (12,13). HIF-1 is a heterodimer consisting of a bHLH-PAS α-subunit and a β-subunit that is a common partner to several other bHLH-PAS proteins. The HIF-1α and HIF-1β subunits are constitutively expressed under normoxia but the α-subunit is rapidly degraded under normoxic conditions via oxygen dependent hydroxylation by proline hydroxylases, followed by recognition and polyubiquitination by pVHL, and subsequent proteolysis. Under hypoxic conditions the α-subunit is stabilized, allowing it to bind to the β-subunit and form active HIF-1, which is then able to bind specific DNA enhancer sequences, known as hypoxia response elements (HREs), and regulate target gene activity (13,18-20).

Two hallmarks of the response to hypoxia are likely due to an attempt to conserve energy: small flies and slower developing flies, which we examine in this study. Flies reared under varying levels of hypoxia, or stressed with hypoxia at any developmental stage, are smaller than wild-type as available oxygen provides a physical constraint on insect body size (11,21-24). Hypoxia also negatively affects cell cycle kinetics resulting in a lengthening of the cell cycle, which slows development (25,26). Additionally, Severe hypoxia (< 3%) directly limits ATP production resulting in lowered rates of protein synthesis, feeding, digestion and absorption (11). Here we investigate the possible link between oxygen starvation, ImpL2 expression, and insulin signaling. ImpL2 transcript abundance was previously reported to be elevated during hypoxia by microarray studies of Drosophila gene expression (27). Our results confirm the previously reported increase in ImpL2 during hypoxia and demonstrate that this upregulation is HIF-1 dependent. Additionally, we show that the ImpL2 transcripts are being actively translated during hypoxia. We find that ImpL2 is required for hypoxic tolerance in adult flies and that it

affects the rate of development in a dosage dependent manner. Coupled with these findings we also show that IIS is repressed under hypoxia and that this repression requires both functional HIF-1 and ImpL2. Thus, hypoxia-induced ImpL2 expression is dependent on HIF-1 and inhibits IIS and promotes hypoxia tolerance.

EXPERIMENTAL PROCEDURES

Fly stocks—The following pre-existing fly stocks from Bloomington Stock Center were used: *Oregon-R*, *Canton S*, *sima*^{7607}, tGPH, *UAS-simaB*, *Lk6-GAL4* for expression in larval fat body, *Elav-GAL4* for expression in embryos and *en-GAL4*.

The laboratory of Ernst Hafen provided the following stocks that were used in this study: *UAS-s.ImpL2* and *ImpL2*^{Def42}*/TM3, Sb*, which was denoted as *ImpL2*$^{+/-}$ in this study (8). Flies listed as *ImpL2*$^{-/-}$ were *ImpL2*^{Def42}*/ImpL2*^{Def42}.

Tissue preparation and immunohistochemistry—Stage 16 embryos were dechorionated and fixed in the standard fashion and antibody staining was performed as described previously (28). In short, embryos were fixed for 20 minutes at 23°C in 4% formaldehyde, blocked for 20 minutes with 5% normal goat serum, and incubated with primary antibodies at 4°C overnight. Secondary antibody incubations were performed at 23°C for 2 hours. Embryos where mounted in glycerol and imaged on confocal microscopes. Feeding 3rd instar larvae were dissected in PBS to expose fat body and then antibody staining was performed as described previously (8).

Molecular Markers—The rat anti-ImpL2 was previously described (15) and provided by J. Natzle (Department of Molecular and Cellular Biology, University of California, Davis, USA). Chicken anti-rat HRP 1:5000 (Santa Cruz Biotechnology),

mouse anti-GFP 1:500 (Molecular Probes), chicken anti-GFP 1:500 (Aves Laboratories, Inc.) and Alexa Fluor® 646 phalloidin 1:200 (Molecular Probes). Secondary antibodies were Alexa Fluor® 488 goat anti-mouse 1:500 (Molecular Probes) and Alexa Fluor® 488 goat anti-chicken 1:500 (Molecular Probes) were also used in this study.

Relative qPCR—Total RNA was isolated using standard TRIzol protocols. cDNA was synthesized following the Super-Script III Reverse Transcriptase protocol (Invitrogen). Real-time PCR was performed using the Sybr Green PCR Master Mix (Applied Biosystems) and a StepOnePlus Real-Time PCR system (Applied Biosystems). Baseline and efficiency were calculated using LinRegPCR software (29). $\Delta\Delta C_t$ values were calculated using StepOnePlus software (Applied Biosystems). The following primers were used: *ImpL2* (5'-TTCGCGGTTTCTGGGCACCC-3' and 5'-GCGCGTCCGATCGTCGCATA-3'), *Actin5c* (5′-GGATGGTCTTGATTCTGCTGG-3' and 5′-AGGTGGTTCCGCTCTTTTC-3') *and Rp49* (5'-ATGACCATCCGCCCAGCATAC-3' and 5'-GAGAACGCAGGCGACCGTTGG-3').

Microscopy—Confocal image stacks were collected using a Leica SP2 or Bio-Rad Radiance 2000 confocal microscope and displayed as two-dimensional projections. Confocal images were processed using ImageJ, Adobe Photoshop CS3 and Adobe Illustrator CS3.

RESULTS

ImpL2 transcript abundance is increased during hypoxia in a HIF-1 dependent manner—Microarray analysis previously revealed a more than four-fold increase in ImpL2 transcript abundance in adult flies in response to 6 hours of hypoxic stress at 0.5%

O_2. By comparing transcript abundance in response to hypoxia for both wild-type and HIF-1α homozygous mutant adult flies using relative qPCR, we verified the previous result and determined that HIF-1 is required for the induction of *ImpL2*. While a four-fold increase is seen between normoxic and hypoxic wild-type adults, no significant difference is seen in *HIF-1α* homozygous mutants (Fig. 1). Therefore, HIF-1 is required for the increase in *ImpL2* in response to hypoxia.

ImpL2 protein is elevated under hypoxic stress—One response to hypoxia is reduced translational activity. Thus, we examined ImpL2 protein levels during hypoxia to determine if the increased transcript abundance was correlated with increased protein levels. First, we confirmed that we could detect ImpL2 protein specifically by examining ImpL2 over-expression embryos (*Elav-GAL4;UAS-s.ImpL2*) compared to wild-type embryos. We used *Elav-GAL4* as a previously characterized pan-neuronal driver with strong expression in embryos (30). Using a previously generated antibody against ImpL2 (8), we found a clear and observable increase in detectable ImpL2 protein in *Elav-GAL4;UAS-s.ImpL2* embryos (Fig. 2A). Next, we examined and observed an increase in the level of ImpL2 protein in response to hypoxia (Fig. 2B). Interestingly, hypoxia consistently resulted in a greater increase in ImpL2 protein levels than pan-neuronal over-expression, suggesting that a greater number and variety of cells were producing ImpL2 protein. Because most translation is shut down under hypoxia (31), the active translation of ImpL2 suggests that ImpL2 is performing an essential function during hypoxic stress.

Increased HIF-1 expression is sufficient for increased ImpL2 protein levels—To test whether HIF-1α is sufficient to induce ImpL2 expression; we examined ImpL2 protein levels in HIF-1α over-expression embryos. Immunohistochemistry in stage 16

embryos revealed that over-expression of HIF-1α (*Elav-GAL4;UAS-simaB*) results in an increase in ImpL2 protein levels (Fig. 2C). Over-expression of HIF-1α is sufficient to induce ImpL2 expression in the absence of any environmental stress. Although HIF-1α is rapidly degraded under normoxia, over-expression is presumably able to override the rate of degradation allowing for HIF-1α to dimerize with HIF-1β and translocate to the nucleus for the activation of target genes. Coupled with the qPCR data, we conclude that HIF-1 is both necessary for the increase in ImpL2 during hypoxia and sufficient to promote its expression during normal conditions.

ImpL2 is required for hypoxic tolerance—While wild-type flies are very tolerant of moderate hypoxia (10% O_2) and can develop fully under these conditions, $ImpL2^{-/-}$ flies raised under the same conditions fail to eclose and many die prior to pupariation (data not shown). Using a survival assay of stressing adult flies with 1.3+/-0.1% for 24 hours, we found that the survival rates of male wild-type, $ImpL2^{+/-}$, $ImpL2^{-/-}$ and $HIF\text{-}1\alpha^{-/-}$ were approximately 75%, 62%, 46% and 42% respectively (Fig. 3). The differences in survival were all statistically significant except when comparing $ImpL2^{-/-}$ and $HIF\text{-}1\alpha^{-/-}$ flies, which showed no significant difference. Even the loss of one copy of *ImpL2* impaired the survival of flies stressed with hypoxia, suggesting that proper levels of ImpL2 are needed for an optimal response to hypoxic stress.

IIS is repressed during hypoxia—To investigate whether IIS is affected during hypoxia we used tGPH flies to assay insulin signaling activity (8). Transgenic tGPH flies express a pleckstrin homology domain: GFP fusion protein, which binds to PIP_3 to visualize IIS activity. When insulin binds the insulin receptor, PI3K phosphorylates PIP_2 to PIP_3 at the cell cortex. Therefore, when insulin signaling is active, PIP_3 accumulates at

the cortex and GFP becomes cortically enriched. In contrast, when insulin signaling is inactive, GFP becomes cytoplasmically diffuse. Thus, immunostaining against GFP allows for visualization of IIS signaling based on cortical localization. We observed that IIS is clearly repressed in the fat body of feeding 3rd instar larvae stressed with 0.5% O_2 for as little as one hour (Fig. 4).

ImpL2 is both necessary and sufficient for the repression of IIS during hypoxia—Examining the location of GFP in the fat body of feeding 3rd instar larvae carrying the tGPH reporter we observed that IIS remains active in *ImpL2* homozygous mutants during hypoxia (Fig. 4). Over-expression of ImpL2 in the fat body of normoxic, feeding 3rd instar larvae using *Lk6-GAL4;UAS-s.ImpL2* flies resulted in silencing of IIS (Fig. 4). Additionally, over-expression of HIF-1α results in IIS silencing (Fig. 4). Thus, both HIF-1 and ImpL2 expression is sufficient to reduce IIS, and ImpL2 is required for the reduction in IIS during hypoxia.

ImpL2 dosage affects rate of development—The average time to develop from embryo to eclosion is 13 days for normoxic wild-type flies and 17 days for hypoxic wild-type flies at 22°C (Fig.5). *ImpL2*$^{-/-}$ flies develop in 11 days and flies over-expressing ImpL2 or HIF-1α require 17-18 days to develop (Fig. 5). Flies unable to inhibit insulin signaling during development could be expected to develop faster while those that experience increased repression of IIS would likely develop slower. This is consistent with the published finding that *ImpL2* mutants are larger than wild-type flies when weights are taken after feeding for 3 days from eclosion.

DISCUSSION

Hypoxia is a severe environmental stress that brings about a host of changes at the organismal, cellular and molecular levels. A key regulator of many of these changes is HIF-1, which alters the transcription of target genes during hypoxia. We have previously shown that targets of HIF-1 regulation extends to controlling the activities of other signaling pathways such as the heat shock response, and thus is able to tune a wide range of cellular activities (32). Here we demonstrate that HIF-1 is required and sufficient for regulating IIS during hypoxia through the increased expression of the IGFBP ImpL2, and that this regulation is a critical aspect of hypoxia tolerance. Thus, these data link together two important pathways that respond to the environment, and show that their coordinated action is needed for an optimal response to low oxygen.

Our results do not support an indirect model of IIS regulation by hypoxia. That is, the increase in ImpL2 and reduction in IIS seen in response to hypoxia could be the result of starvation given that IIS is reduced following nutrient deprivation and adult flies or larvae stressed with severe hypoxia enter a hypoxic stupor and cease feeding (27). However, one hour of severe hypoxia (<1.5% O_2) is sufficient for the complete repression of IIS in larval fat body (Fig. 4) while 4 hours of starvation is required for a similar but less significant effect (8). Second, over-expression of HIF-1 or ImpL2 with the *Lk6-GAL4* driver clearly reduces IIS in the larval fat body during normoxia (Fig. 4) but does not lead to cessation of feeding or development and is therefore comparable to mild hypoxia (Fig. 5) (11). Third, chronic mild hypoxia (5-7% O_2) prolongs development independent of nutritional state as flies continue to feed and grow under these conditions. Fourth, HIF-1 is required for the hypoxic induction of ImpL2 in adults, and sufficient for

the induction of ImpL2 in normoxic embryos (Fig. 1, 2). Furthermore, HIF-1 is sufficient for the reduction of IIS observed in feeding normoxic third instar larvae (Fig 4). We conclude that the increase in ImpL2 and associated silencing of IIS observed during hypoxia is not a result of starvation but a direct result of HIF-1 activity.

While hypoxia and IIS both affect many components of growth and survival and share common target genes, hypoxia has not been previously reported to directly repress IIS (33). We propose a model wherein HIF-1 activation during hypoxia promotes the expression of ImpL2, which acts as an IGFBP and suppresses IIS resulting in slowed development, smaller flies, and hypoxia tolerance. In support of this model we demonstrated that *ImpL2* levels increase in response to hypoxia in a HIF-1 dependent manner, that ImpL2 is required for hypoxia tolerance in a dose dependent manner, that ImpL2 dosage affects development rates, and that hypoxia represses IIS in an ImpL2 dependent manner. Additionally, IIS is known to be silenced during the wandering stage at the end of the 3rd instar as larvae prepare for pupariation; however, IIS remains active during this stage in *ImpL2* homozygous mutants (data not shown). This observation that ImpL2 is required for normal silencing of IIS during wandering as well as in response to environmental stresses implies that ImpL2 is required for proper regulation of IIS during all life stages and is responsible for the developmental delay we observed.

Our data support the conclusion that ImpL2 functions as an IGFBP in order to modulate IIS activity both during normal development and during any time of environmental stress from embryo development through adulthood. Our results also suggest that ImpL2, a putative homolog of vertebrate IGFBP-7, may be the only functional IGFBP in Drosophila as flies are unable to compensate for its loss. The lack of

redundancy in this instance is tolerable because ImpL2 is dispensable under normal conditions with homozygous mutants exhibiting only minor changes in size and developmental rate. Our results further the understanding of the critical role ImpL2 plays in silencing IIS during times of environmental stress to promote survival, and demonstrate the broad role HIF-1 has in orchestrating the activity of multiple cellular pathways in response to stress.

BRIDGE TO CHAPTER III

In the previous chapter I reported data form a paper currently under review for publication, of which I was the first author, which examined the function and regulation of the IGFBP ImpL2 during hypoxia in *Drosophila melanogaster.* We demonstrated that hypoxia-induced ImpL2 expression is dependent on HIF-1, inhibits IIS and promotes hypoxia tolerance. In chapter III I report on my research findings into the regulation and function of another hypoxia-response gene, the bHLH transcription factor *hairy*.

CHAPTER III

THE REGULATION AND FUNCTION OF THE HYPOXIA-RESPONSE GENE *HAIRY* IN ADULT *DROSOPHILA*

Drosophila hairy is a member of the *Hairy and Enhancer of split (HES)* gene family encoding a basic-helix-loop-helix (bHLH) transcription factor that is well characterized for its role as a primary pair-rule gene and negative regulator of *fushi tarazu* (*ftz)* during embryogenesis (34-37). Transcription factors are a class of DNA binding proteins that regulate gene expression. Additionally, *hairy* is a repressor of *achaete* and *scute* during peripheral nervous system development in larvae and pupae. *Hairy* is required for the proper patterning of sensory bristles and derives its name from the mutant phenotype (38-42).

The bHLH transcription factors are a well-conserved yet functionally diverse class of regulatory proteins found in animals, fungi and plants. Much less is known about plant bHLH proteins compared to those found in other eukaryotes (43-46). Different subfamilies of bHLH proteins function as dedicated activators, dedicated repressors or a combination of the two. The primary function of one subclass of bHLH proteins found in both invertebrates and vertebrates, known as Class C bHLH proteins, is the repression of target genes through the recruitment of other non-DNA binding proteins (41,47). The HES family of proteins in *Drosophila* is composed entirely of Class C bHLH proteins. HES proteins contain a basic domain for DNA binding, a Helix-Loop-Helix domain for dimerization, an Orange domain for specificity, and a WRPW motif for binding the

potent co-repressor Groucho. Additionally, hairy contains a domain anterior to the WRPW motif that binds *Drosophila* C-terminal Binding Protein (dCtBP), another co-repressor (Fig. 6) (48-51). HES proteins are required throughout development as transcriptional repressors of genes necessary for a variety of processes including segmentation, neurogenesis and sex determination. Groucho is an essential co-repressor in vertebrates and invertebrates that is known to bind *hairy* and enhance target gene repression (41,52-58). *Hairy* has been well characterized as a dedicated transcriptional repressor in all biological processes it is known to be involved in (59-67).

Hairy was identified by microarray analysis as increasing in transcript abundance in adult *Drosophila* in response to hypoxia, which was subsequently confirmed by semi-quantitative RT-PCR. It was reported that *hairy* is necessary for recovery from hypoxic paralysis and survival under prolonged hypoxia (27). Both the increase in transcript abundance and requirement for hypoxic tolerance supported a previously uncharacterized role for *hairy* in helping adult flies to adapt to and survive hypoxia. With no previous reports of hairy activity outside of embryo and larval development, we were interested in how *hairy* was being regulated during hypoxia in adults, whether or nor it was acting as a transcriptional repressor, and what genes it was regulating. Here I describe my findings regarding the regulation and function of *hairy* during hypoxia.

EXPERIMENTAL PROCEDURES

Fly Stocks—The following pre-existing fly stocks were obtained from Bloomington Stock Center: Oregon R, h^{1}, h^{25}, and h^{41}. In order to minimize differences in gene expression due to variations in the genetic backgrounds of the strains that were

studied, both the wild-type Oregon R control strain and each mutant were crossed to the mapping stock: *yw;Sp/CyO;Dr/TM3Sb*. The genotype of the wild type control strains were *yw;Sp/CyO;+/+* and *yw;Sp/CyO;+/TM3Sb.* The genotypes of the mutant strains studied were as follows: *yw;Sp/CyO;*h^1/h^1*, yw;Sp/CyO;*h^{25}*/TM3Sb,* and *yw;Sp/CyO;*h^{41}*/TM3Sb.*

Adult Hypoxia Treatment—15 male and 15 female adult flies form each genotype between the ages of 3 to 7 days post-eclosion were anaesthetized with CO_2 and placed in separate vials containing standard *Drosophila* medium for each hypoxia experiment. After a 24-hour recovery period vials of the wild type strain and each mutant were placed in a sealed chamber at 22°C that was then flushed with a mixture of 0.5% O_2 and 99.5% N_2 (severe hypoxia). The other vials for each respective genotype were placed in an identical chamber at 22°C containing normal atmospheric oxygen concentrations. The flies were incubated under either hypoxia or normoxia for six hours. Following the treatment, the flies were removed quickly and immediately frozen in liquid nitrogen.

RNA Extraction and Microarray Experiments—Following either hypoxia or normoxia treatment, frozen flies were homogenized in TRIzol reagent and RNA was extracted according to the manufacturer's protocol (Invitrogen). For each strain, 20 μg of RNA from each normoxia and hypoxia treatment were labeled for microarray hybridization using the SuperScript Direct cDNA Labeling System (Invitrogen), incorporating either Cyanine 3 or Cyanine 5 conjugated dUTP (Perkin Elmer). In each strain, gene expression during hypoxia was assayed by combining labeled normoxia and hypoxia cDNA, and hybridizing the samples to DNA microarrays containing 16,416 oligonucleotides from the INDAC set representing the *D. melanogaster* transcriptome

(Illumina). Microarrays were scanned and analyzed using Gene Pix Pro 6.0 software (Molecular Devices). We then examined the expression level of transcripts in wild-type flies compared to the *hairy* mutant strains in order to identify genes that were differentially regulated during hypoxia in order to identity possible *hairy* targets.

Cell Culture and Hypoxia Treatments—*Drosophila melanogaster* Kc_{167} and S2 tissue culture cells were obtained from the Drosophila Genomics Resource Center (DGRC). Cells were maintained in Schneider's *Drosophila* medium (Invitrogen) supplemented with 5% (for Kc_{167} cells) or 10% (for S2 cells) heat-inactivated fetal bovine serum (Invitrogen). For hypoxia experiments, cells were incubated for 6 hours at 22°C in chambers flushed with 0.5% O_2 and 99.5% N_2. Following the treatment, total RNA was isolated using standard TRIzol protocols. The synthesis of cDNA and microarray experiments were perfomed as described above.

RNA Interference (RNAi)—RNAi was performed as previously described (68). The following primer pairs were used to generate template DNA: control green fluorescent protein (GFP) (5'-GCCACAAGTTCAGCGTGTCC-3' AND 5'-GCTTCTCGTTGGGGTCTTTC-3'), *hairy* (5'-CCGACTGTGTGAACGAGGTTAGC-3' and 5'-TGACCGATGGCTTGATGTCC-3'). The T7 promoter sequence (5'-TAATACGACTCACTATAGGGAGA-3') was added to the 5' end of all the above primers when ordered (IDT).

Hypoxia Reporter Construction—A small region of the *hairy* gene containing putative HREs was cloned into into the p-Green H Pelican reporter vector. Kc_{167} cells were then transfected with the recombinant reporter plasmid using the Effectene®

Transfection Reagent Kit (Qiagen) and then either left under normoxia or placed under hypoxia as described above.

RESULTS

The hairy gene region contains conserved putative HREs—We used a bioinformatic approach to search for putative HIF-1 binding sites in the *hairy* gene region. Since HIF-1 is the master regulator of the hypoxic response it is the prime candidate for regulation of any hypoxia-response gene. Additionally, we have repeatedly observed an increase in *hairy* transcript in response to hypoxia in both adults and *Drosophila* Kc cells, and this increase appears to be HIF-1 dependent as HIF-1α RNAi in Kc cells reduces the increase in hypoxia induced hairy transcription and a similar result is seen in HIF-1α mutant flies (Unpublished data). *Hairy* contains multiple putative HREs that are conserved in the genomes of several Drosophila species (Fig. 7).

The putative HREs near hairy are not bound by HIF-1 during hypoxia—We next tested whether the HREs had a regulatory function during hypoixa. Having identified a cluster of conserved putative HREs upstream of hairy we cloned them upstream of a minimal promoter driving GFP in the p-Green H Pelican reporter vector (69). We then co-transfected Kc cells with this reporter vector and a puromycin resistance vector. After selecting for stable transfectants we placed the cells under normoxic or hypoxic conditions. If the HREs were functionally relevant then the hypoxic cells would have shown a dramatic increase in GFP fluorescence compared with normoxic cells. The hypoxic cells transfected with the reporter vector did not show an increase in GFP fluorescence, suggesting they are not functionally relevant, and therefore, not bound by

HIF-1. As a control, Kc cells were transfected with a reporter vector containing a portion of the second intron of *heat shock factor* (*Hsf*) which is know to include functionally relevant HREs (32). The hypoxic cells containing this reporter showed a dramatic increase in GFP fluorescence compared to normoxic cells.

The transcriptional response of hairy mutants to hypoxia is the same as in wild-type flies—DNA microarrays were used to compare the transcriptional responses of both wild-type and *hairy* mutant adult *Drosophila melanogaster* that had been stressed with hypoxia with those who had remained under normoxic conditions. Only transcripts exhibiting a fold change of 0.59 or greater on the log2 scale were considered. On a log2 scale, 0.59 ($2^{0.59}$) is equivalent to a 2-fold change in transcript abundance so only genes with at least a 2-fold increase were evaluated in this study. After exposure to hypoxic stress, wild-type flies exhibited a 2-fold or greater increase in 164 transcripts while h*airy* mutants exhibited an increase in 208 transcripts. Although more genes increased in transcript abundance from exposure to hypoxia in *hairy* mutants than in wild-type flies, the overall transcriptional responses were surprisingly similar (Table 1).

Since *hairy* is considered a dedicated transcriptional repressor, we expected to see a significant increase in transcript abundance in a subset of genes in the *hairy* mutants with the reasoning being that they were no longer under repression. On the contrary, microarrays with hairy mutants produced results surprisingly similar to controls. We were limited to working with either heterozygous amorphs (h^{25} and h^{41}) or a homozygous hypomorph (h^{1}), as *hairy* null alleles are homozygous lethal. After performing microarray experiments with both classes of mutants we concluded that perhaps there was not a

sufficient reduction in hairy protein to see a clear effect on target genes so we decided to try RNAi in *Drosophila* Kc cells.

RNAi based silencing of hairy in Kc cells results in no significant transcriptional changes during hypoxia compared to controls—DNA microarrays were used to compare the transcriptional responses of control Kc cells and Kc cells treated with hairy-RNAi that had been stressed with hypoxia with those who had remained under normoxic conditions. We confirmed knock-down (KD) of hairy transcript abundance in cells treated with hairy-RNAi with semi-quantitative RT-PCR. Although the actual number of genes that changed in transcript abundance varied between controls and hairy-KD cells, the overall transcriptional responses were very similar for important genes of known function (Table 2). Only the top 200 transcripts are displayed for each group of cells. Microarray experiments did not result in the identification of any clear candidate genes as Hairy targets during hypoxia.

Putative Hairy targets previously identified by DamID increase in transcript abundance during hypoxia—A previously published paper reported the use of a chromatin profiling technique known as DamID to identify direct targets of Hairy (42). In this technique an *E. coli* adenine methyltransferase (DAM) tethered to a chromatin binding protein leads to specific methylation of DNA adjacent to the protein binding sites (70-72). When we compared the findings from the DamID experiments to our microarray results we identified six genes that were differentially expressed during hypoxia that were also identified as putative direct targets of Hairy: *kayak*, *ImpL2*, *astray*, *Smg5*, CG13868 and CG15745. Additionally, all six genes contain conserved Hairy binding sites with the motif CACGCG. Surprisingly, all five genes increase in transcript abundance during

hypoxia, which would not be expected of the direct targets of a dedicated transcriptional repressor; on the contrary, we would expect to see those genes downregulated. There is no significant change in the hypoxic response for four of the six aforementioned genes in *hairy* mutants. The two genes that are expressed differently in hairy mutants (Smg5 and CG15745) actually show diminished transcriptional upregulation in response to hypoxia.

DISCUSSION

A better understanding of the regulation and function of the *Drosophila* gene *hairy* is important because of the critical role the mammalian homologues (HES1-6) play during many stages of human development and in tumorogenesis. HES1 is the primary mammalian homologue of *hairy* but all of the HES genes share a common structure and function as transcriptional repressors that are crucial components in the regulation of many biological processes including neurogenesis, eye development, somite segmentation, neural stem cell maintenance, embryonic stem cell differentiation, adult and pediatric tumor metastasis, and glia lineage restriction of neural progenitors in the injured or diseased central nervous system (73-81). This study proposes a novel role for *hairy* in promoting hypoxia tolerance in adult *Drosophila melanogaster* and identifies six putative hypoxia-response targets, one of them being the IGFBP ImpL2 described in Chapter II.

BRIDGE TO CHAPTER IV

In the previous chapter I provided background on the function of bHLH proteins as a class of transcription factors found in all metazoa. Additionally, I described the results of my investigations into the regulation and function of the hypoxia-response gene *hairy*, a homologue of the mammalian HES1 gene. In Chapter IV I will summarize the findings from Chapters II and III and highlight the broader significance of these results.

CHAPTER IV

CONCLUSION

Insulin/Insulin-Like growth factor signaling (IIS) plays a critical role in regulating many aspects of normal development including, but not limited to, proliferation, body size, metabolism, fertility, and longevity. Mis-regulation of IIS is associated with diabetes miletus, numerous human cancers and an increased risk of all-cause death in men and women alike. A better understanding of the molecular mechanisms underlying IIS is imperative for advancements in the treatment of various developmental disorders and diseases. *Drosophila melanogaster* has proved to be an invaluable model organism for unraveling many biological mysteries related to human development and disease with discoveries in flies very often being applicable to humans. Approximately 50% of *Drosophila* protein sequences have human homologues and 75% of human disease genes have *Drosophila* counterparts or homologues.

In chapter II I reported on significant findings I made that elucidated both the regulation and function of the hypoxia-response gene *ImpL2*. Using relative qPCR I confirmed that the transcriptional increase of *ImpL2* during hypoxia that was previously observed in microarrays and demonstrated that the increase in *ImpL2* transcript abundance was dependent upon HIF-1. Using antibodies to ImpL2 in embryos I determined that the *ImpL2* transcripts were being translated during hypoxia and that HIF-1 over-expression was sufficient to promote an increase in ImpL2 protein levels during normoxia thus supporting a regulatory role for HIF-1. By comparing survival rates of

wild-type and *ImpL2* mutants to hypoxic stress I demonstrated that ImpL2 was required for hypoxia tolerance in adult flies. Using transgenic tGPH flies I was able to determine that IIS is repressed in the fat body of feeding 3rd instar larvae during hypoxic stress and that ImpL2 was both necessary and sufficient for the repression of IIS during hypoxia. Finally, by comparing the developmental rates of wild-type, ImpL2 mutant, and ImpL2 over-expression flies I observe that ImpL2 dosage affects the rate of development. My finding supported my initial hypothesis that activation of HIF-1 during hypoxia promotes the expression of ImpL2, which in turn functions as an IGFBP to inhibit IIS and promote hypoxia tolerance.

In chapter III I reported on the results of my investigation into the regulation and function of another hypoxia-response gene, *hairy*. While *hairy* proved to be more enigmatic than *ImpL2*, I was able to show that while there are multiple putative HREs in the *hairy* gene region, they do not appear to be directly bound by HIF-1 during hypoxia. Additionally, using microarrays to assay the entire Drosophila transcriptome during normoxia and hypoxia, I demonstrated that anything short of a complete loss of gene product was insufficient to alter the transcriptional response of adult flies or cells in culture. And finally, I was able to identify six putative direct targets of Hairy that changed in transcript abundance during hypoxia. This was puzzling, however, since Hairy is considered a dedicated transcriptional repressor while my findings suggest it may be acting as a transcriptional activator. Of particular interest was the finding that Hairy is implicated in the regulation of ImpL2.

APPENDIX A

FIGURES WITH LEGENDS FOR CHAPTER II

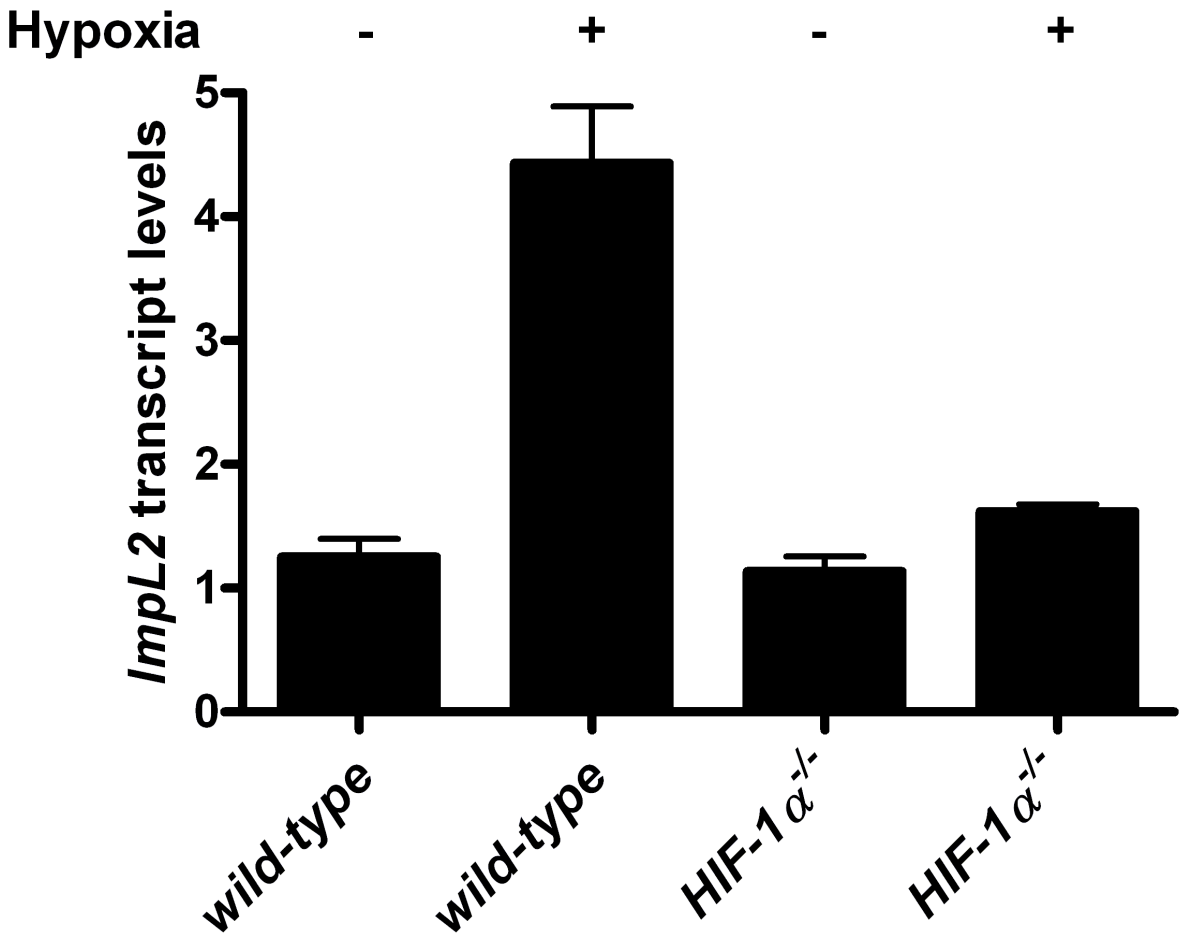

Fig. 1. ImpL2 transcription is elevated during hypoxia in a HIF-1 dependent manner. Quantification of ImpL2 transcript levels by relative qPCR both confirms the increase in transcript abundance previously reported by microarray and reveals that HIF-1α is required for the increase in ImpL2 transcript during hypoxia. While there is significantly more ImpL2 in hypoxic wild-type flies than in normoxic flies, there is no significant difference in ImpL2 levels between normoxic and hypoxic HIF-1α homozygous mutants.

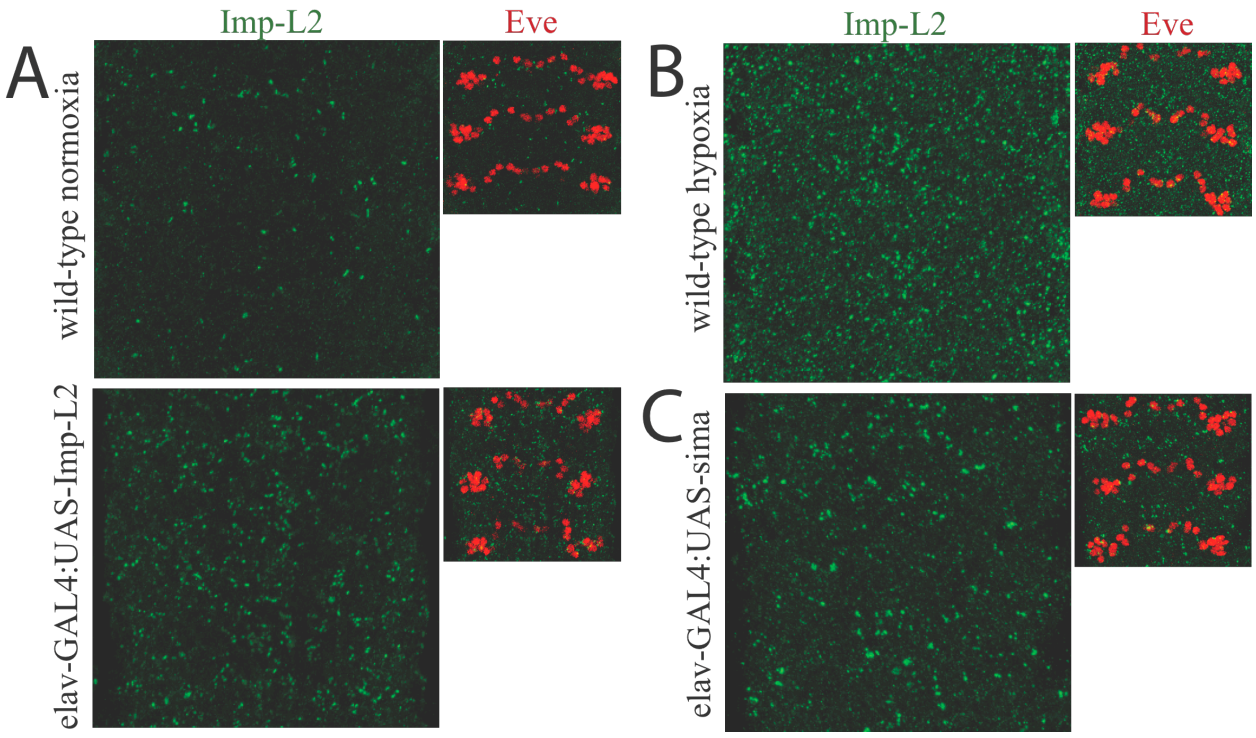

Fig. 2. ImpL2 protein is elevated during hypoxia. A. Antibody detection in wild-type embryos confirms a detectable increase in ImpL2 protein levels when over-expressed with a pan-neuronal GAL4 driver. ImpL2 is labeled in green and the EVE^{+} U1-U5 motorneurons are labeled in red as a control for imaging depth. B. A considerable increase in ImpL2 protein levels is also observed during hypoxia. C. Over-expression of sima (Drosophila HIF-1α) using the same pan-neuronal driver as before results in an increase in ImpL2 protein levels in normoxic embryos. All panels show two segments of a stage 16 embryo. Panels are presented as Z-projections covering the U1-U5 motorneurons (~ 20 um). N > 20 for all experiments.

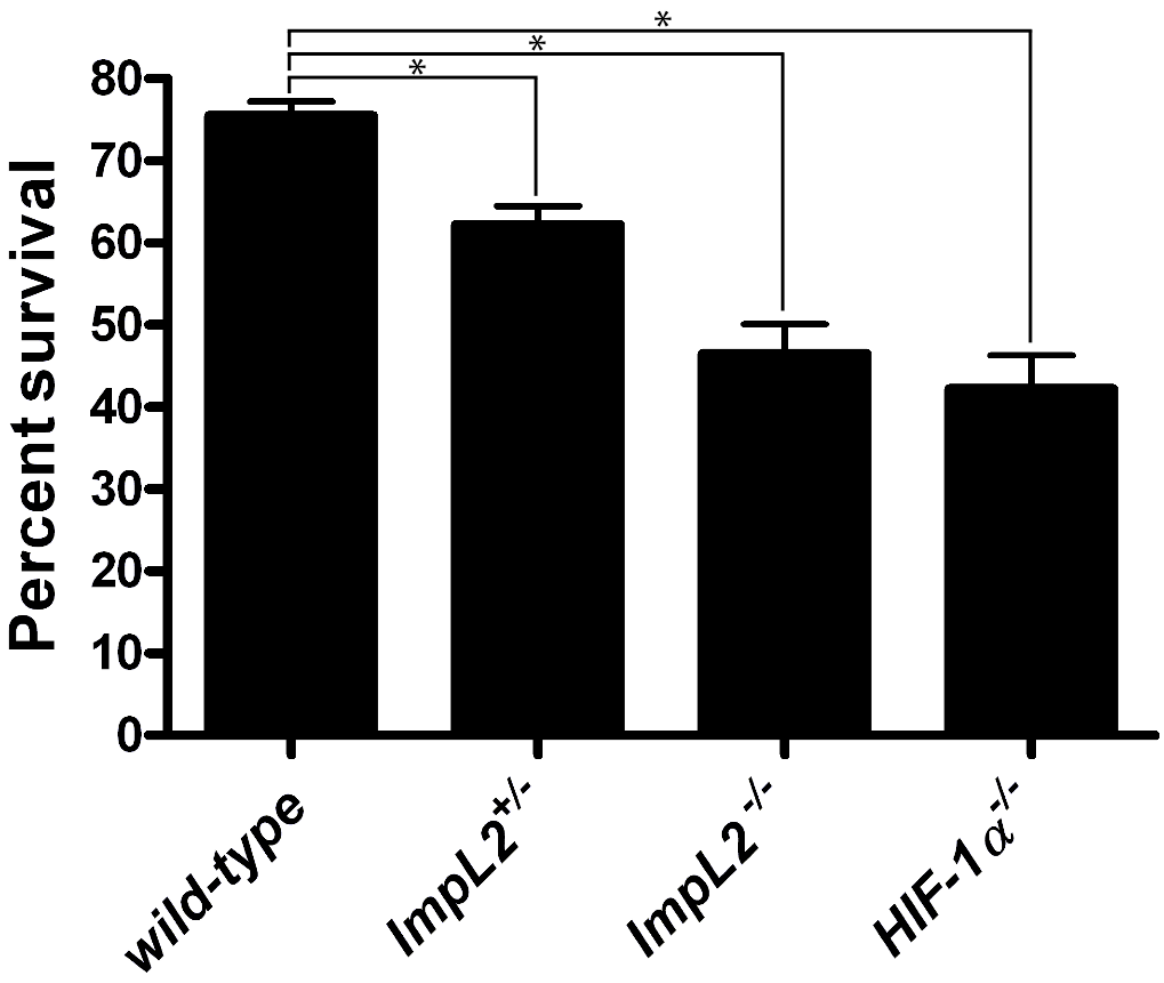

Fig. 3. ImpL2 is required for hypoxic tolerance in adult flies. Flies missing one copy of ImpL2 are more sensitive to hypoxia. Additionally, flies missing both copies have a survival rate no different than HIF-1α homozygous mutants. Reductions in survival between $ImpL2^{+/-}$, $ImpL2^{-/-}$, and wild-type are statistically significant. * All p-values are 0.005 or less. Error bars represent standard error of the mean. Results shown are from four biological replicates.

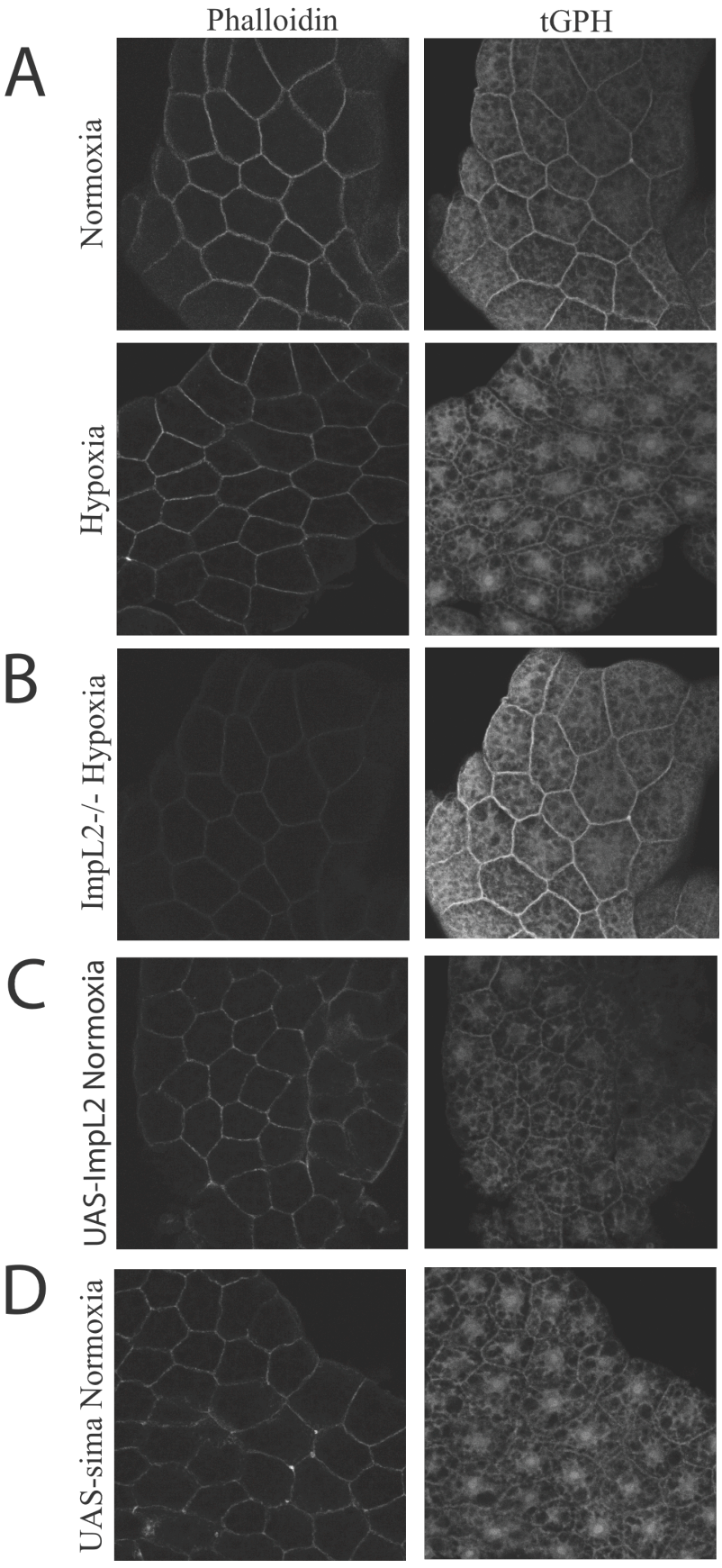
Phalloidin
tGPH
A
Normoxia
Hypoxia
B
ImpL2-/- Hypoxia
C
UAS-ImpL2 Normoxia
D
UAS-sima Normoxia

Fig. 4. ImpL2 is necessary for the repression of IIS during hypoxia in larval fat body. Phalloidin staining is shown in the left column to reveal the cell membrane. tGPH fluorescence, a reporter for IIS activity, is shown in the right column under varying conditions. A. Cortical enrichment of tGPH in wild-type feeding third instar larvae indicates normal IIS activity. IIS activity is repressed during hypoxia as evidenced by the loss of cortical localization of tGPH. B. tGPH expression in ImpL2 homozygous mutants during hypoxia is similar to expression in wild-type normoxic larvae, indicating active IIS. C, D. IIS activity is repressed during normoxia when either ImpL2 or sima (Drosophila HIF-1α) is over-expressed in the larval fat body using the Lk6-Gal4 driver.

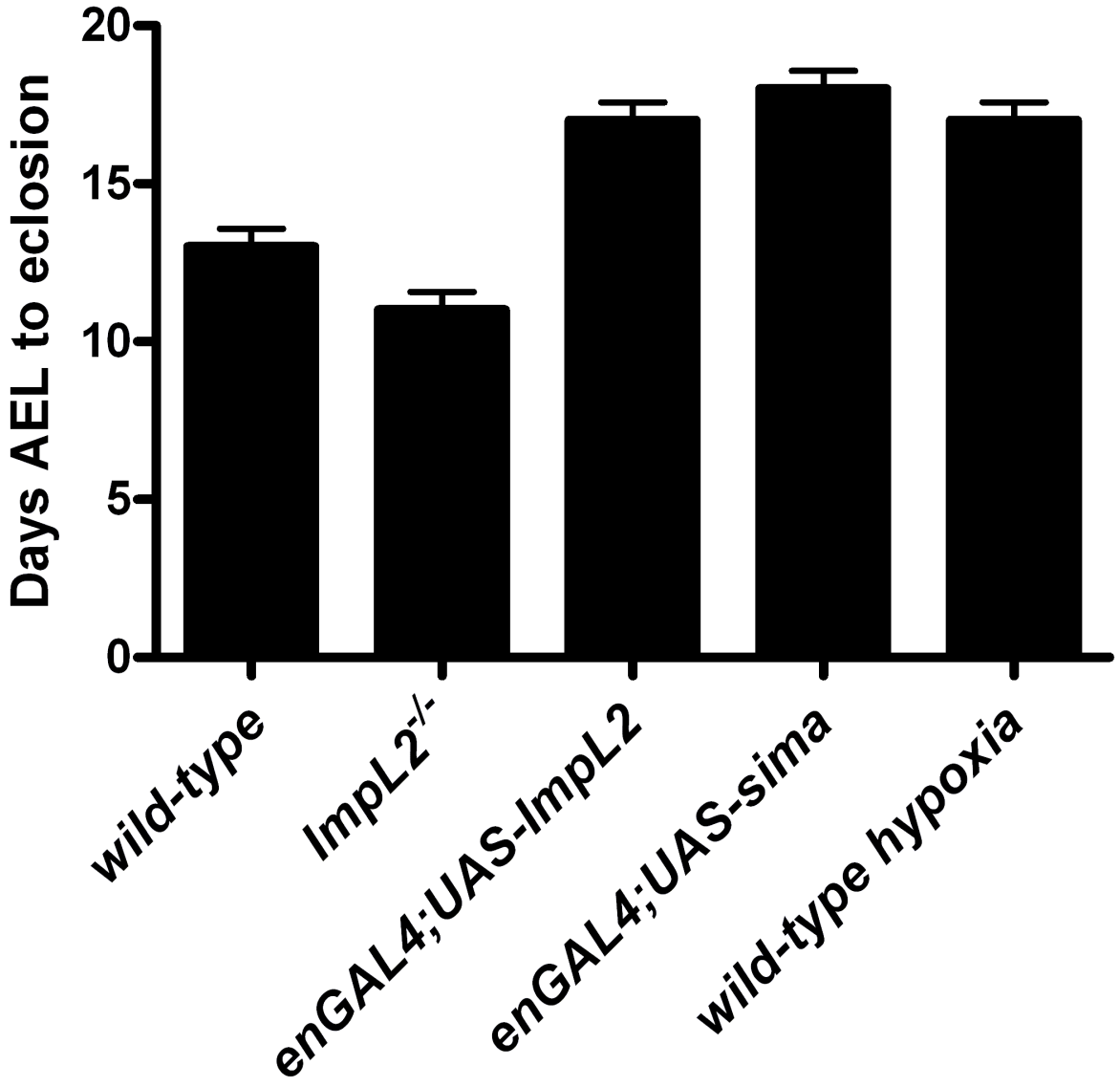

Fig. 5. ImpL2 dosage affects rate of development. Wild-type flies reared at 22°C eclose after approximately 13 days after egg laying. In contrast, ImpL2$^{-/-}$ flies develop 2 days faster, while over-expression of ImpL2 slows development by 4 days. In a similar manner, over-expression of sima (Drosophila HIF-1α) slows development by 5 days. The slower rate of development is also observed in flies raised under mild hypoxia (10% O_2).

APPENDIX B

FIGURES WITH LEGENDS AND TABLES FOR CHAPTER III

Fig. 6. Essenstial domains of the Hairy protein**.** Hairy, like all HES proteins, contains a basic domain (b) for DNA binding, a Helix-Loop-Helix (HLH) domain for dimerization, an Orange domain for specificity, a domain for binding the co-repressor *Drosophila* C-terminal Binding Protein (dCtBP), and a WRPW motif for binding the potent co-repressor Groucho.

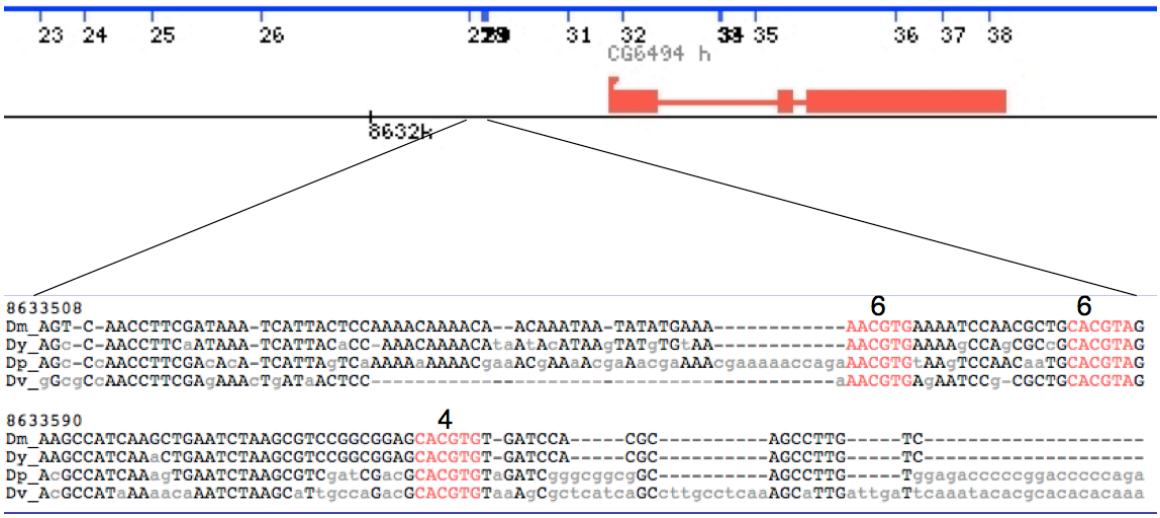

Fig. 7. Putative HREs in the *hairy* gene region. There are 3 highly conserved putative HREs upstream of the promoter region of the hypoxia-response gene *hairy*. Only 4 of the 12 sequenced Drosophila genomes are displayed. Putative HRE's are in red and the numbers above represent how many Drosophila species have conservation in these regions.

TABLE 1: Fold increases in transcript abundance in response to hypoxia in both wild-type and *hairy* mutant adult *Drosophila*.

Wild-type

Fold Change	Transcripts
3.88	CG18743 Heat-shock-protein-70Ab
3.81	CG31449 Heat-shock-protein-70Ba
3.67	CG5834 Hsp70Bbb
2.96	CG4463 Heat shock protein 23
2.61	CG10160 Ecdysone-inducible gene L3
2.49	CG32130
2.38	CG5436 Heat shock protein 68
2.38	CG32041 Heat shock gene 67Bb
2.35	CG12896
2.25	CG5550
2.22	CG11825
2.20	CG4181 Glutathione S transferase D2
2.06	CG15154 Suppressor of cytokine signaling at 36E
1.86	CG11765 Peroxiredoxin 2540
1.83	CG15009 Ecdysone-inducible gene L2
1.83	CG15009 Ecdysone-inducible gene L2
1.69	CG17725 Phosphoenolpyruvate carboxykinase
1.68	CG10383
1.65	CG8846 Thor
1.63	CG9434 Frost
1.63	CG41040
1.60	CG5592
1.59	CG10578 DnaJ-like-1
1.58	CG16898
1.56	CG31769
1.55	CG18816 Tetraspanin 42Eb
1.55	CG13517 Odorant-binding protein 59a
1.52	CG17524 Glutathione S transferase E3
1.49	CG5953
1.47	CG13321
1.47	O49366_1
1.47	CG6494 hairy
1.44	CG13868
1.44	CG30160
1.43	CG7219
1.43	CG15678
1.40	CG11086
1.40	CG5408 tribbles
1.40	CG8709
1.39	CG16978
1.39	CG11796
1.38	CG15784
1.38	CG7224
1.36	CG17325

1.33	CG3884
1.28	CG15829
1.28	CG10078 Phosphoribosylamidotransferase 2
1.25	CG16926
1.25	CG32106
1.25	CG18466 Nmdmc
1.23	CG8330 tomboy40
1.21	CG13490
1.19	CR31541
1.19	CG9127 adenosine 2
1.19	CG11652
1.11	CG10073
1.09	CG15423
1.09	CG12489 Defense repressor 1
1.08	Q9LU32_1
1.08	CG15893
1.08	CG14207 HSP20-like chaperone 18D3
1.05	CG12242 Glutathione S transferase D5
1.05	CG6687
1.05	CG13713
1.05	CG32597
1.04	CG1552]
1.03	CG31543 HIF prolyl hydroxylase
1.02	CR31400 Heat shock RNA &ohgr
1.00	CG18279 Immune induced molecule 10
1.00	CG7130
0.99	CG7850 puckered
0.99	CR31400 Heat shock RNA &ohgr
0.97	CG32071
0.97	CG16844 Immune induced molecule 3
0.97	CG4779 homogentisate 1,2-dioxygenase
0.96	CG15673
0.95	CG2789
0.95	CG4533 lethal (2) essential for life
0.94	CG5059
0.92	CG15043
0.92	CG5164 Glutathione S transferase E1
0.92	CG10031 Protease inhibitor I2
0.90	CR32989 U6atac snRNA at 29B
0.90	CG7554 comm2
0.89	CG11992 Relish
0.89	CG3090 Sox box protein 14
0.88	CG18106 Immune induced molecule 2
0.87	CG2914 Ets at 21C
0.87	CG3705 astray
0.87	CG14935
0.87	CG4427 cabut
0.87	CG16713
0.86	CG15389 eyes shut

0.85	CG8913
0.85	CG32103
0.85	CG30489 Cyp12d1-p
0.84	CG13511
0.84	CG5295
0.84	CG4067 pugilist
0.84	CG11967
0.82	CG12946
0.81	CG14933 Proteinase inhibitor I1
0.80	CG1049 CTP:phosphocholine cytidylyltransferase 1
0.80	CG4472 Imaginal disc growth factor 1
0.80	CG2471
0.80	CG31764 virus-induced RNA 1
0.80	CG8808 Pyruvate dehydrogenase kinase
0.79	CG6945
0.78	CG1333 Ero1L
0.77	CG11992 Relish
0.77	CG18330 CTP:phosphocholine cytidylyltransferase 2
0.76	CG17533 Glutathione S transferase E8
0.75	CG4183 Heat shock protein 26
0.75	CG17836
0.75	CG14383
0.74	CG4312 Metallothionein B
0.72	CG8954 Smg5
0.72	CG5748 Heat shock factor
0.72	CG12643
0.72	CG1817 Protein tyrosine phosphatase 10D
0.72	CR32905 snoRNA:Z1
0.72	CG3989 ade5
0.72	CG7590 scylla
0.71	CG3546
0.71	CG1921 sprouty
0.71	CG9453 Serine protease inhibitor 4
0.71	CG4899 Photoreceptor dehydrogenase
0.71	CG8501
0.71	CG15509 kayak
0.71	CG18233
0.70	CG1924
0.70	CG4919 Glutamate-cysteine ligase modifier subunit
0.70	CG3896
0.69	CG3365 drongo
0.69	CG10641
0.69	CG1014 robl62A
0.68	CG3884
0.68	CG15745
0.68	CG7005 Epidermal stripes and patches
0.68	CG2275 Jun-related antigen
0.68	CG10660
0.66	CG5571

0.65	CG1882
0.65	CG8127 Ecdysone-induced protein 75B
0.64	CG18317
0.64	CG1583
0.63	CG12437 raw
0.63	CG10962
0.63	CG10360 refractory to sigma P
0.63	CG12065
0.63	CG10045 Glutathione S transferase D1
0.62	CG31957
0.62	CG13510
0.62	CG1630 Inositol 1,4,5-triphosphate kinase 2
0.62	CG5399
0.61	CG10245 Cyp6a20
0.61	CG15658
0.61	CG1921 sprouty
0.60	CG10600
0.60	CG2928 Rhythmically expressed gene 5
0.60	CG6761
0.60	CR32894 snoRNA:U29:54Ec
0.59	CG13279 Cytochrome b5-related
0.59	CG13689

Hairy Mutant

Fold Change	Transcripts
4.47	CG18743 Heat-shock-protein-70Ab
4.12	CG31449 Heat-shock-protein-70Ba
4.10	CG5834 Hsp70Bbb
3.41	CG4463 Heat shock protein 23
3.41	CG5436 Heat shock protein 68
3.16	CG32041 Heat shock gene 67Bb
2.66	CG32130
2.66	CG4181 Glutathione S transferase D2
2.55	CG10160 Ecdysone-inducible gene L3
2.12	CG5550
2.06	CG11825
2.05	CG15154 Suppressor of cytokine signaling at 36E
1.94	CG9434 Frost
1.88	CG12896
1.86	CG11765 Peroxiredoxin 2540
1.83	CG15678
1.78	CG17325
1.73	CG16978
1.72	CG8846 Thor
1.69	CR32905 snoRNA:Z1
1.67	CG10078 Phosphoribosylamidotransferase 2
1.64	CG15009 Ecdysone-inducible gene L2
1.63	CG10578 DnaJ-like-1
1.61	CG17524 Glutathione S transferase E3

1.61 CG11086
1.60 CG14027 Turandot M
1.51 CG15784
1.51 CG5953
1.48 CG15423
1.47 CG17725 Phosphoenolpyruvate carboxykinase
1.47 CG10383
1.45 CG13321
1.44 CG15009 Ecdysone-inducible gene L2
1.43 CR31400 Heat shock RNA &ohgr
1.41 CG6494 hairy
1.41 CG17531 Glutathione S transferase E7
1.37 CG7224
1.37 CG18466 Nmdmc
1.36 CG13868
1.36 CG14207 Heat shock protein Hsp20 HSP20-like chaperone 18D3
1.33 CG15829 Acyl-coA-binding protein
1.31 CG5408 tribbles
1.31 CR32896 snoRNA:snR38:54Eb
1.29 CG8709 Lipin
1.26 CR31400 Heat shock RNA &ohgr
1.26 CG18816 Tetraspanin 42Eb
1.24 CG7219
1.24 CG6687
1.23 CG4533 lethal (2) essential for life
1.22 CG31769
1.21 CG3884
1.19 CG4312 Metallothionein B
1.17 CG18106 Immune induced molecule 2
1.16 CR32894 snoRNA:U29:54Ec
1.16 CG31957
1.15 CG4183 Heat shock protein 26
1.15 CG15043
1.14 CG1552
1.12 CG15893
1.12 CG30160
1.11 CG11652 Diphthamide synthesis
1.11 CG16926
1.11 CG2789
1.10 CG12643
1.10 CG11796
1.10 CG11992 Relish
1.09 CG4608 branchless
1.09 CG16844 Immune induced molecule 3
1.08 CG11967
1.06 CG5592
1.05 CG15848 Sarcoplasmic calcium-binding protein 1
1.05 CG13947
1.04 CG5164 Glutathione S transferase E1

1.04	CG9127 adenosine 2
1.01	CG18330 CTP:phosphocholine cytidylyltransferase 2
1.00	CG2914 Ets at 21C
1.00	CG6945
0.98	CG31764 virus-induced RNA 1
0.98	CG9568
0.98	CG8330 tomboy40
0.97	CG33192 Metallothionein D
0.96	CG16713 Protease inhibitor I2
0.96	CG18108 Immune induced molecule 1
0.96	CR31541
0.94	CG1367 Cecropin A2
0.94	CG12489 Defense repressor 1
0.93	CG18001 Ribosomal protein L38
0.93	CG4067 pugilist
0.93	CG3090 Sox box protein 14
0.93	CG5346
0.93	CG4427 cabut
0.92	CG31451
0.92	CG7850 puckered
0.92	CG12946
0.92	CG3896
0.92	CG33486 asparagine synthetase
0.90	CG4472 Imaginal disc growth factor 1
0.90	CG15673
0.89	CR32989 U6atac snRNA at 29B
0.89	CG10045 Glutathione S transferase D1
0.88	CG10041 Peptidase S1
0.88	CG7554 comm2
0.88	CG16775
0.87	CG14933 Proteinase inhibitor I1
0.86	CG4899 Photoreceptor dehydrogenase
0.85	CG10570
0.84	CG8764 oxen
0.84	CG3705 astray
0.84	CG4618
0.84	CR32881 snRNA:U5:38ABa
0.84	CG14801
0.84	CG31543 HIF prolyl hydroxylase
0.83	CG18107
0.83	CG7130
0.83	CG8095 scab
0.82	CG32106
0.81	CG5461 bunched
0.81	CR32874 snoRNA:U25:30E
0.80	CG17836
0.79	CG31811 centaurin gamma 1A
0.79	CG15068
0.78	CG3348

0.78 CG13057 retinin
0.78 CG15509 kayak
0.78 CG2981 Troponin C at 41C
0.78 CG15065
0.77 CG8622 Accessory gland-specific peptide 53Ea
0.77 CG11089
0.77 CG32952
0.77 CG4779 homogentisate 1,2-dioxygenase
0.76 CG17673 Accessory gland peptide 70A
0.76 CG11992 Relish
0.76 CG3884
0.76 CG32767
0.75 CG10962
0.75 CG16712 Protease inhibitor I2
0.75 CG1049 CTP:phosphocholine cytidylyltransferase 1
0.75 CG8913
0.75 CR32865
0.74 CG14935
0.74 CG30473 Odorant-binding protein 51a
0.73 CG4241 alternative testis transcripts open reading frame A
0.73 CG5059
0.73 CG8808 Pyruvate dehydrogenase kinase
0.73 CG32529
0.72 CG10852 Accessory gland peptide 63F
0.72 CG1361 Andropin
0.72 CG33495 Ductus ejaculatorius peptide 99B
0.71 CG2471
0.71 CG4802
0.71 CG1921 sprouty
0.70 CG5571
0.70 CG5399
0.70 CG17239 proteolysis Peptidase S1
0.69 CG12065
0.69 CG4986 Male-specific RNA 57Dc
0.69 CG1882
0.69 CG12876
0.69 CG7590 scylla
0.69 CR32862 small nuclear RNA U1 at 82Eb
0.69 CG10031 Protease inhibitor I2
0.68 CG3943 kraken
0.68 CG32041 Heat shock gene 67Bb
0.67 CG5295
0.67 CG2330
0.67 CG3986 Chitinase 4
0.67 CG3871 Six4
0.67 CG10580 fringe
0.66 CG12845 Tetraspanin 42Ef
0.66 CG15658
0.66 CG17533 Glutathione S transferase E8

0.65	CG6761
0.65	CG13116
0.64	CG3529
0.64	CG14542
0.64	CG18317
0.64	CR32665 RNA on the X 2
0.64	CG32137
0.64	CG8369
0.64	CG7188
0.64	CG9285 Dipeptidase B
0.63	CG4898 Tropomyosin 1
0.63	CG31549
0.63	CG15547
0.63	CR32895 snoRNA:U29:54Ed
0.63	CG7033
0.62	CG8127 Ecdysone-induced protein 75B
0.62	CG16836
0.62	CG3640
0.62	CG5352 Small ribonucleoprotein particle protein B
0.62	CG9415 X box binding protein-1
0.62	CG16704
0.62	CG7390 Senescence marker protein-30
0.62	CG18233
0.61	CG31468
0.61	CG32103
0.61	CG1044 daughter of sevenless
0.61	CR40454 5.8SrRNA:CR40454
0.61	CG14724 Cytochrome c oxidase subunit Va
0.60	CG3989 ade5
0.60	CG10365
0.60	CG31704 Proteinase inhibitor I1
0.60	CG1921 sprouty
0.60	CG6416
0.59	CG12775 Ribosomal protein L21
0.59	CG32039
0.59	CG5958
0.59	CG15064 Him

TABLE 2: Fold increases in transcript abundance in response to hypoxia in both control cells and *hairy*-RNAi cells.

Control cell

Fold Change	Transcripts
3.87	CG8846 Thor
3.52	CG10160 Ecdysone-inducible gene L3
3.18	CG7224
2.51	CG4726
2.46	CG1552
2.37	CG15009 Ecdysone-inducible gene L2
2.34	CG15009 Ecdysone-inducible gene L2
2.33	CG8330 tomboy40
2.31	CG10746 fledgling of Klp38B
2.31	CG3348
2.28	CG5729 Dgp-1
2.26	CG18743 Heat-shock-protein-70Ab
2.14	CG31449 Heat-shock-protein-70Ba
2.05	CG41040
2.04	CG31543 HIF prolyl hydroxylase
1.90	CG31299 nocturnin
1.89	CG5834 Hsp70Bbb
1.85	CG7331
1.82	CG2017
1.81	CG10242
1.80	CG1882
1.79	CG1621
1.74	CG6494 hairy h
1.73	CG3767 Juvenile hormone-inducible protein 26
1.71	CG1014 robl62A
1.71	CG32041 Heat shock gene 67Bb
1.68	CG32464 l(3)82Fd
1.66	CR31541
1.62	CG3050 Cyp6d5 C
1.53	CG3424
1.49	CG17299 SNF4/AMP-activated protein kinase
1.49	CG1435 sarcoplasmic calcium-binding protein CBP
1.47	CG32369
1.47	CG10833
1.45	CG32464 l(3)82Fd
1.44	CG4351
1.43	CG4427 cabut
1.41	CG18135
1.38	CG5164 Glutathione S transferase E1
1.37	CG32512
1.36	CG17278
1.36	CG10011
1.35	CG3476
1.34	CG2789

1.34 CG3001 Hexokinase A
1.33 CG7130
1.32 CG33254
1.31 CG4583 ire-1
1.31 CG4863 Ribosomal protein L3
1.30 CG3821 Aspartyl-tRNA synthetase
1.29 CG6608
1.29 CG3714
1.29 CG12800 C
1.27 CG18578 Ugt86Da
1.26 CG4832 centrosomin
1.23 CG11050
1.22 CG31811 centaurin gamma 1A
1.22 CG15154 Suppressor of cytokine signaling at 36E
1.20 CG3017 Aminolevulinate synthase
1.19 CG10802
1.18 CG16987 Activin Like Protein at 23B
1.18 CG12358 polyA-binding protein interacting protein 2
1.16 CG14005
1.15 CG17534 Glutathione S transferase E9
1.15 CG7234 Glutamate receptor IIB
1.14 CG11652
1.13 CG15784
1.11 CG5968
1.11 CG14709
1.11 CG6428
1.11 CG2928 Rhythmically expressed gene 5
1.09 CG11372 galectin
1.08 CG4840
1.08 CG4608 branchless
1.07 CG4909 Plenty of SH3s
1.07 CG11779
1.07 CG11143 Inos
1.06 CG13117
1.05 CG12534
1.05 CG17342 Lk6
1.05 CG33486 asparagine synthetase
1.03 CG10360 refractory to sigma P
1.03 CG5748 Heat shock factor Hsf
1.03 CG5789
1.02 CG13624
1.02 CG10990
1.02 CG7841
1.02 CG12317 JhI-21
1.02 CG17836
1.01 CG11086
1.01 CG3825
1.01 CG7239
1.01 CG3061 C

1.01	CG18317
1.00	CG33131 SCAP
0.99	CG14478
0.99	CG32233
0.98	CG7737
0.98	CG1135
0.97	CG30022
0.96	CG40100
0.96	CG30023 sprite
0.96	CG6815 belphegor
0.95	CG7717 Mekk1
0.94	CG1333 Ero1L
0.94	CG10660
0.94	CG17724
0.92	CG7044
0.92	CG3395 Ribosomal protein S9
0.92	CG32217 Su(Tpl)
0.92	CG13517 Odorant-binding protein 59a
0.92	CG12389 Farnesyl pyrophosphate synthase
0.92	CG10315 eIF2B-&dgr
0.91	CG5517 Insulin degrading metalloproteinase
0.90	CG4944 ciboulot
0.90	CG6778 Glycyl-tRNA synthetase
0.90	CG3168
0.89	CG3458 Topoisomerase 3&bgr
0.89	CG8198 lethal (1)
0.89	CG2791
0.88	CG7235
0.87	CG7761 parcas
0.87	CG2803 Troponin C-akin-1
0.87	CG17533 Glutathione S transferase E8
0.86	CG1921 sprouty
0.85	CG9821
0.85	CG17531 Glutathione S transferase E7
0.85	CG7590 scylla
0.85	CG33267
0.84	CG32103
0.83	CG7664 cropped
0.83	CG12425
0.83	CG4183 Heat shock protein 26
0.82	CG33123
0.80	CG33005
0.80	CG15675
0.80	CG5247 Inverted repeat-binding protein
0.80	CG9773
0.80	CG6115
0.79	CG7668
0.79	CG11188
0.79	CG5010

0.78 CG32529
0.78 CG32742 lethal (1)
0.78 CG33075 Tyler
0.78 CG8531
0.78 CG8913
0.78 CG6721 GTPase-activating protein 1
0.78 CG8233
0.77 CG7954 steamer duck
0.77 CG1620
0.77 CG12223 Dorsal switch protein 1
0.77 CG8251 Phosphoglucose isomerase
0.77 CG3845 lethal (2)
0.77 CG9066
0.77 CG7565
0.76 CG3782 mitochondrial ribosomal protein L28
0.76 CG6843
0.76 CG5874
0.76 CG10497 Syndecan
0.76 CG3090 Sox box protein 14
0.76 CG5165 Phosphogluconate mutase
0.76 CG10321
0.76 CG2865
0.75 CG33162 Signal recognition particle receptor &bgr
0.75 CG5535
0.75 CG8443
0.75 CG4311 HMG Coenzyme A synthase
0.75 CG6155 Roe1
0.74 CG5654 ypsilon schachtel
0.74 CG10778
0.74 CG30035
0.74 CG3059 NTPase
0.74 CG3806 eIF2B-&egr
0.74 CG5820 Gp150
0.73 CG10383
0.73 CG1676 cactin
0.72 CG8026
0.72 CG13645
0.72 CG10535
0.72 CG11560
0.71 CG5231 Lipoic acid synthase
0.71 CG9779
0.70 CG4589
0.70 CG30437
0.70 CG1921 sprouty
0.70 CG11471 Isoleucyl-tRNA synthetase
0.70 CG7085 lethal (2) s5379
0.70 CG7602 DNApol-&igr
0.70 CG8188
0.69 CG8190 eIF2B-&ggr

0.69	CG5151
0.69	CG7283 Ribosomal protein L10Ab
0.69	CG8893 Glyceraldehyde 3 phosphate dehydrogenase 2
0.69	CG5041 Tfb4
0.69	CG18596
0.68	CG17064 mars
0.68	CG6808
0.68	CG10578 DnaJ-like-1
0.68	CG7611 WD-40 repeat Lissencephaly type-1-like homology motif

Hairy-RNAi

Fold Change	Transcripts
4.10	CG31449 Heat-shock-protein-70Ba
4.07	CG18743 Heat-shock-protein-70Ab
4.04	CG5834 Hsp70Bbb
2.92	CG4183 Heat shock protein 26
1.83	CG32041 Heat shock gene 67Bb
1.78	CG3348
1.67	CG8846 Thor
1.61	CG4463 Heat shock protein 23
1.58	CG10160 Ecdysone-inducible gene L3
1.34	CG4726
1.21	CG2341 Ccp84Ad
1.09	CR32905 snoRNA:Z1
1.01	CG7224
1.01	CG4982
0.96	CG3395 Ribosomal protein S9
0.92	CG31617 His1:CG31617
0.92	CG33486 asparagine synthetase
0.90	CG40373
0.82	CG5164 Glutathione S transferase E1
0.82	CG31299 nocturnin
0.82	CG40322
0.80	CG11086 Ribosomal protein L7Ae/L30e/S12e/Gadd45
0.79	CG7283 Ribosomal protein L10Ab
0.78	CG7130
0.77	CG15154 Suppressor of cytokine signaling at 36E
0.75	CG4114 expanded
0.74	CG5729 Dgp-1
0.73	CG14632
0.73	CG32369
0.72	CG1882
0.71	CG11050
0.69	CG7602 DNApol-&igr
0.69	CG10578 DnaJ-like-1
0.67	CR32887 snoRNA:U31:54Ea
0.67	CG1474 Es2
0.67	CR32894 snoRNA:U29:54Ec
0.67	CG4863 Ribosomal protein L3

0.66 CG5748 Heat shock factor
0.65 CG11652
0.63 CG32143
0.62 CG8620
0.60 CG32143
0.56 CR31614 His-&PSgr
0.56 CG2791
0.56 CG5231 Lipoic acid synthase
0.56 CG7235
0.54 CR32899 snoRNA:U31:54Ed
0.51 CG31543 HIF prolyl hydroxylase
0.51 CG17531 Glutathione S transferase E7
0.50 CG17534 Glutathione S transferase E9
0.49 CG17533 Glutathione S transferase E8
0.49 CG18317
0.49 CG14005
0.48 CR32162 snRNA:U12:73B
0.48 CG12379
0.48 CG10778
0.48 CG32491 modifier of mdg4
0.47 CG9888 Fibrillarin
0.46 CG33229
0.46 CG3476
0.45 CG2986 overgrown hematopoietic organs at 23B
0.45 CG1621
0.44 CG13117
0.44 CG17023 Dead box protein 80
0.44 CG6746
0.44 CG10746 fledgling of Klp38B
0.43 CG5317
0.43 CG2017
0.43 CG11802
0.42 CG9075 Eukaryotic initiation factor 4a eIF-4a
0.42 CG2116
0.42 CG5840
0.42 CG5231 Lipoic acid synthase
0.41 CG9895
0.40 CG17167
0.40 CG3806 eIF2B-&egr
0.40 CG4195 lethal (3) 73Ah
0.39 CG17759 G protein &agr
0.38 CG31811 centaurin gamma 1A
0.37 CG13136
0.37 CG10318 NC2&agr
0.37 CG1890
0.36 CG3821 Aspartyl-tRNA synthetase
0.36 CG32510
0.36 CG17737
0.35 CG8261 G protein &ggr

0.35 CG7603
0.35 CG33005
0.35 CG17153
0.35 CG14096
0.35 CG8675
0.34 CG12317 JhI-21
0.34 CG17299 SNF4/AMP-activated protein kinase gamma subunit
0.34 CG11188
0.34 CG41072
0.33 CG7737
0.33 CG1584 Origin recognition complex subunit 6
0.33 CG17765
0.33 CG33199
0.33 CG15012
0.33 CG2803 Troponin C-akin-1
0.32 CG6401
0.32 CG17510
0.32 CG8580 bhringi
0.32 CG18578 Ugt86Da
0.32 CG7954 steamer duck
0.32 CG9089 wurst
0.32 CG3495 GDP-4-keto-6-deoxy-D-mannose 3,5-epimerase/4-reductase
0.31 CG10423 Ribosomal protein S27
0.31 CG8359
0.31 CG17187
0.31 CG1721 Phosphoglyceromutase
0.31 CG32474 dysfusion
0.31 CG32068
0.31 CG9066
0.31 CG1467 Syntaxin 16
0.30 CG3260 Zinc finger protein RP-8
0.30 CG8229
0.30 CG5446 Heat shock factor binding 1 33E4
0.30 CG3714
0.29 CG40451
0.29 CG15009 Ecdysone-inducible gene L2
0.29 CG4813
0.29 CG40084
0.29 CG14066 La related protein
0.29 CG16969
0.29 CG12945
0.28 CG10306
0.28 CG12278
0.28 CG4800 Translationally controlled tumor protein
0.28 CG14715
0.27 CG1319
0.27 CG40224
0.27 CG5268 black pearl
0.27 CG3017 Aminolevulinate synthase

0.27 CG18593 viral IAP-associated factor
0.27 CG15514
0.27 CG12795
0.27 CG12173
0.27 CG8860
0.27 CG4214 Syntaxin 5
0.26 CG13521 roundabout
0.26 CG15929 lin-52
0.26 CG3767 Juvenile hormone-inducible protein 26
0.26 CG2608
0.26 CG6038
0.26 CG5224
0.26 CG5056
0.26 CG5861
0.25 CG30415
0.25 CG7772
0.25 CG2674 Minute (2) 21AB
0.25 CG32672 Autophagy-specific gene 8a
0.25 CG8206
0.25 CG12295
0.25 CG4591 Tetraspanin 86D
0.25 CG5057 Mediator complex subunit 10
0.25 CG33184 mitochondrial ribosomal protein L53
0.25 CG13688 Ipk2
0.24 CG1715 lethal (3) 03670
0.24 CG11500
0.24 CG6608
0.24 CG5629
0.24 CG1088 Vacuolar H+-ATPase 26kD E subunit
0.24 CG6155 Roe1
0.24 CG6335 Histidyl-tRNA synthetase
0.24 CG17903 Cytochrome c proximal
0.24 CG10360 refractory to sigma P
0.24 CR31615 His-&PSgr
0.24 CG5941
0.24 CR32957
0.24 CG31865
0.23 CG31492
0.23 CG7885 RNA polymerase II 33kD subunit
0.23 CG40274
0.23 CG9836
0.23 CG18217
0.23 CG12265
0.23 CG17166
0.23 CG33249
0.23 CG3034 Mediator complex subunit 22
0.23 CG7713
0.23 CG11331 Serpin-27A
0.23 CG6115

0.23	CG6151
0.23	CG9035 Translocon-associated protein &dgr
0.23	CG15812 piefke
0.23	CG5134 Mediator complex subunit 9
0.23	CG5497 mitochondrial ribosomal protein S28
0.22	CG7850 puckered puc
0.22	CG8053 Eukaryotic initiation factor 1A eIF-1A
0.22	CG5994 Negative elongation factor E
0.22	CG17023 Dead box protein 80
0.22	CG9669
0.22	CG31360
0.22	CG10851 B52
0.22	CG11984
0.22	CG8636
0.22	CG11110
0.22	CG5395

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