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Purification and characterization of antibodies against killifish HIF-1α

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DEDICATION

This thesis is dedicated to my parents, my husband and kids, who have supported me along the journey.

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ABSTRACT

Many fish face low oxygen concentrations (hypoxia) in their natural environments, and they respond to hypoxia through a variety of behavioral, physiological, and cellular mechanisms. Some of these responses involve changes in gene expression. In mammals, the hypoxia inducible factor (HIF) family of transcription factors are the "master regulators" of gene expression during hypoxia, but the study of HIF in fish has been hampered by the lack of reagents to detect this protein in non-mammalian vertebrates. The goals of this thesis are to affinity purify antibodies against HIF from the killifish *Fundulus heteroclitus* and use them to recover and quantify HIF from killifish cells and tissues. Purified, validated antibodies represent a critical reagent for future studies of the role of HIF in the molecular response of this and other fish to fluctuations in oxygen in their natural environments.

Key words: antibodies, cell line, *Fundulus grandis, Fundulus heteroclitus,* HIF-1α, hypoxia, hypoxia inducible factor, and immunoprecipitation

1. INTRODUCTION

1.1 Oxygen

Energy transformations are a defining characteristic of life. In animals, the energy required to support activities, such as growth, locomotion, and reproduction, is obtained by the degradation of complex foodstuff molecules, generally through the process of aerobic metabolism. In aerobic metabolism, the production of energy depends upon oxygen as the final acceptor of electrons in mitochondrial electron transport. When cellular oxygen levels are low (hypoxia) or absent (anoxia), other pathways of energy production may be invoked, but these yield less energy per molecule of starting material and are limited in duration by the availability of fermentable substrates and the accumulation of waste products (Hochachka and Somero, 2002). For those cells, organs, and animals that rely upon aerobic metabolism for the bulk of their energy, therefore, a deficiency of oxygen is associated with decreased energy production, cellular dysfunction, organ impairment, and, if not reversed, death.

Because of oxygen's central role in aerobic metabolism, metazoans have evolved a suite of responses to ensure adequate oxygen delivery to tissues, or when these do not meet tissue energy requirements, to tolerate periods of tissue hypoxia (Hochachka and Somero, 2002; Semenza, 2014). These responses may be behavioral (increased ventilation rate), physiological (redistribution of blood flow to critical tissues), cellular (new blood vessel growth), or biochemical (increased glucose transport into and utilization by cells). Many of these responses depend on changes in gene expression, for example increased expression of erythropoietin (EPO) to signal red blood cell maturation, vascular endothelial growth factor (VEGF) to stimulate new blood vessel growth, and glucose transporters and glycolytic enzymes to support increased glucose metabolism. Thus, elucidating the control of gene expression in response to low oxygen

is key to understanding the ability of animals to survive periods of oxygen deprivation.

1.2 Hypoxia-Inducible Factors

One response in mammals to low oxygen is a dramatic increase in the synthesis of erythropoietin (EPO). This glycoprotein hormone stimulates the maturation of red blood cells, and its activity is important in ensuring adequate red blood cell number during blood loss or tissue hypoxia. In studies of the physiological regulator of EPO synthesis, Semenza and Wang (1992) described a protein factor that was induced by hypoxia that bound to a regulatory region of the EPO gene and increased its expression. This protein was named hypoxia inducible factor-1 (HIF-1). Subsequently, HIF-1 was found to be expressed in cells that do not function in erythropoiesis and, in those cells it regulates the expression of other genes involved in the hypoxia response of mammalian cells (Semenza, 2009). Moreover, HIF-1 is one member of a family of transcription factors that include other members (HIF-2 and HIF-3) (Kaelin, 2005). As a group, the hypoxia-inducible factors (HIFs) are now referred to the "master regulators" of oxygen homeostasis in mammals (Semenza, 2010).

HIF-1 is a heterodimer whose subunits are basic helix-loop-helix (bHLH) Per-ARNT-Sim (PAS)-family proteins (Semenza, 1998). The α subunit is oxygen regulated and the β subunit is constitutively expressed (Semenza, 1999). HIF-1β was previously described as the aryl hydrocarbon receptor nuclear translocator (ARNT) and plays a central role in the cellular response to hydrocarbon pollutants (McIntosh *et al*., 2010). The protein structure of both subunits is characterized by bHLH and PAS domains in the N-terminal half that are involved in dimerization of the protein subunits and binding to target DNA (Figure 1) (Gradin *et al.*, 1996; Minet *et al.*, 1999). Oxygen sensitivity of the α subunit is localized within the oxygen-dependent

degradation (ODD) domain and the expression of target genes is regulated by the transactivation domain (TAD) near the C-terminus (Semenza, 2000).

Under normoxia, or normal oxygen tension, prolyl hydroxylase domain enzymes (PHD) hydroxylate proline residues in the ODD domain. Hydroxylation promotes the interaction between the α subunit and the von Hippel-Lindau ubiquitin ligase complex that marks the α subunit for degradation by the 26S proteasome (Figure 2) (Huang *et al.*, 1998; Maxwell *et al.*, 1999; Kaelin, 2005). In addition, another hydroxylase, factor-inhibiting HIF (FIH), modifies an asparagine residue in the C-TAD (Mahon, 2001). This modification prevents HIF from recruiting the coactivator proteins p300 and CREB binding protein (CBP), thereby limiting gene expression by HIF, which might escape degradation (Kaelin, 2005).

When oxygen levels drop, proline and asparagine hydroxylation are inhibited due to the lack of oxygen and the HIF-α subunit is stabilized (Semenza, 2000). HIF-α accumulates (Jewell *et al.*, 2001), dimerizes with HIF-β (Kallio *et al.*, 1998), and recruits p300/ CBP. In the nucleus, the complex binds to regulatory DNA regions, hypoxia response elements (HREs), of specific genes (Wenger and Gassmann, 1997) and activates their transcription. The result is changes in the expression of genes that enhance oxygen delivery to tissues or increase the cellular tolerance of low oxygen.

1.3 The Aquatic Environment

In mammals, low oxygen levels at the tissues occur during development and normal physiology (e.g., strenuous exercise), or in a variety of pathological conditions (e.g., stroke, ischemia, certain cancers). Except for high altitudes and subterranean burrows, however, mammals and other air-breathing vertebrates respire air with a relatively high and constant oxygen content. This is not true of water-breathing animals, including fish. Oxygen is only about

$1/30^{th}$ as soluble in water as it is in air. In addition, oxygen solubility in water decreases as temperature or salinity increases (Wetzel, 2001). Also, the diffusion rate of oxygen in water is $1/10{,}000^{th}$ its diffusion rate in air. The physical properties of oxygen in water result in wide fluctuations in dissolved oxygen concentration when the processes that deplete oxygen and the process that produce or replenish it are not balanced.

Oxygen is absorbed by water by diffusion from the atmosphere or produced by photosynthesis. Photosynthetic oxygen production depends upon photoperiod, water clarity, and the abundance of plants and phytoplankton. On the other hand, oxygen is consumed by chemical and biological processes, notably respiration of the organisms in the water column and on the bottom of the channel or basin. (Wetzel, 2001). In many aquatic habitats, therefore, the concentration of dissolved oxygen peaks during the day and declines at night, in some cases taking overnight dissolved oxygen values close to zero (Wetzel, 2001). In addition to diurnal cycles, dissolved oxygen can vary with tides and season, and in some cases hypoxia persists throughout the year (Wetzel, 2001; Rabalais *et al*., 2010).

Aquatic hypoxia has existed throughout geologic time, and fish and other aquatic organisms have evolved a range of adaptations to variable oxygen concentration. This make fish ideal systems for the study of hypoxia responses. In addition, aquatic hypoxia has intensified in estuarine and coastal areas due to human activities in the last 25 years (Diaz and Rosenberg, 1995; Rabalais *et al*., 2010), in some cases exceeding the capacity of fish to tolerate low oxygen. In these cases, mass mortality or "fish kills" occur. More subtle, non-lethal responses have also been documented, for example poor growth or reproduction (Pollock *et al*., 2007). Some species are more tolerant of low oxygen, presumably through compensatory mechanisms, which may include changes in gene expression, possibly regulated by hypoxia inducible factors.

1.4 Fish and HIF

Although most of what is known about HIF has been learned from studies of mammals in the context of disease, the literature demonstrates the existence of HIF in fish and suggests that it plays a role in the response of fish to ecological hypoxia (Nikinmaa and Rees, 2005; Richards, 2009). Like mammals, fish have three forms of HIF, HIF-1, HIF-2, and HIF-3, which are likely orthologs of the mammalian genes (Rytkonen *et al*., 2011). The regulation of HIF levels in fish has received some attention, and there are some similarities, as well as some important differences when compared to mammals. Among the differences is that in several species, the mRNA levels of HIF-1α appear to be influenced by oxygen levels rather than being oxygen-independent. For example, in sea bass, HIF-1α mRNA was highly expressed in the liver after 4 h hypoxia exposure (DO 1.9 mg/l) (Terova *et al*., 2008). In the same study, after a chronic hypoxia exposure for 15 days, mRNA copy number was also higher in liver but lower in the muscle, kidney, brain, and heart compared with normoxic controls (Terova *et al*., 2008). In Atlantic croaker, ovarian HIF-1α mRNA levels increases within 12 h hypoxia exposure (DO 1.7 mg/l) (Rahman and Thomas, 2007). In a field study, Atlantic croaker collected from hypoxic sites in the Gulf of Mexico showed elevated HIF-1α mRNA expression in ovarian tissue compared to fish collected from normoxic sites (Thomas and Rahman, 2009). In other species, however, mRNA levels are not altered by hypoxia. In rainbow trout gonad cells (RTG-2) and Chinook salmon embryonic cells (CHSE-214), HIF-1α mRNA expression did not change during a 2 and 4 h hypoxia exposure (DO ~ 0.4 mg/l) (Soitamo *et al*., 2001). In Wuchang bream, levels of HIF-1α mRNA varied among tissues under normoxic conditions, but there were no significant changes in HIF-1α mRNA in liver and kidney tissue taken from hypoxic fish (DO 1mg/l, 4 h) (Shen *et al*., 2010).

Less is known about HIF protein levels in fish during the transition from normoxia to hypoxia. The original report of HIF-1α in fish cells showed an increase in protein abundance at modest levels of hypoxia and normoxic degradation was blocked by an inhibitor of the proteasome (Soitamo *et al*., 2001). In another study, accumulation of HIF-1α protein was detected after a 6 h hypoxia exposure using nuclear protein extracts from crucian carp gills (Sollid *et al.,* 2006). Law *et al.* (2006) reported that HIF- 1α protein was detected in liver of normoxic grass carp and expression increased after 4 and 24 h hypoxia exposures. In general, the lack of reagents to reliably detect HIF-α protein in fish has resulted in a fragmentary picture of HIF protein abundance, regulation, and function in fish.

1.5 Antibodies

Polyclonal antibodies are secreted by different B-cell lineages with the body. They are a collection of immunoglobulin molecules that recognize multiple epitopes on a specific antigen. The antibodies are produced after immunization of goat, mouse, rabbit, or chicken. Often antibodies are tagged with reporter molecules such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) so they can be detected by light or color changes.

Immunized hens transfer immunoglobulins (Ig) from the serum to the egg yolk, leading to the designation IgY (Leslie and Clem, 1969). They differ from mammalian antibodies by molecular mass, structure, and where they occur (in yolk rather than plasma). As a non-mammalian species, chickens offer many advantages over conventional antibody production using mammalian species (Camenisch, 1999). Chicken housing is inexpensive, egg collection is noninvasive, and low quantities of antigen are required to attain high IgY titers in egg yolk (Gassmann *et al.*, 1990; Hatta *et al.*, 1993; Song *et al.*, 1985). The amount of IgY harvested from a week's worth of eggs is significantly greater than antibodies acquired from rabbit blood

collected during an equivalent period (Gassmann *et al.*, 1990). Camenisch *et al.* (1999) generated a polyclonal HIF-1α antibody in chicken against the human HIF-1α. They validated that affinity purified HIF-1α antibody was successful in detecting HIF-1α protein from various mammals in many applications.

1.6 *Fundulus* as a Model System

Fundulus heteroclitus (Atlantic killifish or the mummichog) is a small teleost found along the Atlantic coast of North America. The sister species *Fundulus grandis* (Gulf killifish) is native to the Gulf of Mexico and the Atlantic coast of Florida to Cuba. These fishes inhabit brackish and coastal waters. *F. heteroclitus* and *F. grandis* are non-migratory fish, thriving best at warm temperatures (Fangue *et al*., 2009; Brown *et al*., 2011). Their ability to tolerate a variety of environmental conditions, including wide fluctuation in temperature, salinity, and oxygen concentration, make these species popular scientific models for studying physiological and biochemical responses to varying environmental conditions (Burnett *et al*., 2007). In particular, several studies indicate that both *F. heteroclitus* and *F. grandis* tolerate lower levels of dissolved oxygen than many other salt marsh fish species (Cochran and Burnett, 1996; Love and Rees, 2002; Stierhoff *et al*., 2003; Wannamaker and Rice, 2000). Other characteristics that make *F. heteroclitus* a good model system include a sequenced genome and the availability of cultured cells (Gignac *et al*., 2014). In addition, *F. heteroclitus and F. grandis* are abundant in nature and are easy to maintain in the laboratory.

1.7 Research Goals

The first goal of this thesis is to purify antibodies against HIF from the killifish *Fundulus heteroclitus*. This goal will be addressed by cloning, expressing, and purifying a recombinant protein corresponding to a fragment of the *F. heteroclitus* HIF-1α, and using it to affinity purify

antibodies specific for this protein. The second goal of this thesis is to use these antibodies to quantify HIF-1α in killifish cells and tissues. To this end, the technique of immunoprecipitation will be optimized and applied to killifish cells and tissues subjected to normoxia, hypoxia, or cobalt chloride ($CoCl_2$), a hypoxic mimetic. Purified, validated antibodies represent a critical reagent and immunoprecipitation is a key technical approach that will enable future studies of the role of HIF in the molecular response of this and other fish to fluctuations in oxygen in their natural environments.

2. MATERIALS AND METHODS

2.1 Anti-HIF-1α IgY

Chicken polyclonal antibodies were generated by Aves Labs against a recombinant fragment of HIF-1α from *Fundulus heteroclitus* (Townley *et al*., in prep). The fragment corresponds to amino acids 406-510 (ELQPQDCLYDLLKEQPDALTLLAPAAGDMIISLDFSRPETEPQLLKDVPLYSDVMLPSA DDKLALPLSPLSPTEPLEASSCEEAKPDGFAPAVSTSPPRKPSDVD) which has a calculated molecular mass of 11.3 kD. All experiments reported in this thesis used IgYs purified by the vendor from eggs collected from hen #6210.

2.2 Cloning, Expression, and Purification of Polyhistidine-tagged HIF-1α Peptide

To generate polyhistidine-tagged HIF-1α peptide (104 amino acids), the pETDuet vector system (Novagen) was used. Approximately 2 μg of HIF-1α/pcDNA generated by Townley *et al.* (in prep) and 0.2 μg of pETDuet were digested at 37°C in 20 μl reactions containing 2μl 10X NEBuffer 3 (1X Buffer Components**:** 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.2 μl 100X BSA (10 mg/ml), 1 μl Sal-1 (20,000 units/ml; NEBiolabs), and 1 μl of Pst-1 (20,000 units/ml; NEBiolabs). Sal-1 was added first and the reactions incubated for one hour. Then Pst-1 was added and reactions were incubated for an additional hour. Products were separated by gel electrophoresis in 0.8% agarose (Seakem LE Agarose; Cambrex) in 1X TAE Buffer (0.04 M Tris-acetate, 0.001 M EDTA) and visualized by post staining in 1μg/ml ethidium bromide (Fisher Biotech) in 1X TAE buffer. The migration of digests was compared against a 2-log DNA ladder (NEBiolabs). The fragment corresponding to the partial HIF-1α cDNA (312 bp) and digested pETDuet vector (5400 bp) were excised and purified (Geneclean; MP Biomedicals).

The HIF-1α partial cDNA and pETDuet DNA were ligated at a molar ratio containing approximately 40 ng HIF-1α DNA and 50 ng pETDuet DNA (14.4:1 molar ratio). The reaction contained 1X ligase buffer (NEBiolabs), 400 units of T4 ligase (NEBiolabs) and DNA in a final volume of 20 µl. The reaction was incubated at 14°C for 18 h. The HIF-1α/pETDuet recombinant plasmid from the ligation was transformed into *E.coli* DH5α competent cells (Invitrogen). DH5α cells (50 µl) were allowed to thaw on ice, after which 5 µl of each ligation reaction were added to separate tubes of cells. The tubes were mixed gently, returned to ice, and incubated for 30 min. The tubes were heated at 42°C for 30 s then returned to ice for 2 min after which 400 µl room temperature SOC medium (EMD Millipore) was added to each tube. Tubes were incubated for 1 h at 37°C. Two volumes of cells for each ligation combination (40 µl and 400 µl) were spread onto LB agar plates containing ampicillin (100 µg/ml; MP Biomedicals) and incubated at 37°C for 18 h. Colonies were selected and used to inoculate 5 ml cultures in LB containing ampicillin. Cultures were grown for 18 h at 37°C while shaking at 220 rpm. StrataPrep Plasmid Miniprep Kits (Agilent Technologies) were used to purify HIF-1α/pETDuet plasmid from *E.coli* cultures.

Ligation was confirmed by PCR amplification of plasmids using an MJ Mini Personal Thermal Cycler (BioRad) programed for 30 cycles (30 s at 95°C, 30 s at 55°C, 30 s at 72°C). PCR was carried out using 25 µl reactions containing 0.5 µl forward primer pETUpstream (10 µM) 5'-ATGCGTCCGGCGTAGA-3′ (Novagen), 0.5 µl reverse primer DuetDOWN1 (10 µM) 5'-GATTATGCGGCCGTGTACAA-3′ (Novagen), 13 µl PCR master mix (2X) (Promega), 1 µl of plasmid DNA, and 10 µl RNase free water. PCR products were verified by electrophoresis (as described previously).

Ligations were also verified by restriction enzyme digestion of plasmids. Each reaction contained 15.6 μl of plasmid (~850 - 1000 ng plasmid DNA of which 50-55 ng is the HIF-1α insert), 2.2 μl NEBuffer 3 (10X), 0.22 μl 100X BSA (10 mg/ml), 1 μl Sal-1 (20,000 units/ml) and 1 μl Pst-1 (20,000 units/ml). Reactions incubated for 2 hours at 37°C. Restriction digest products were verified by electrophoresis (as described previously).

The inserts were also verified by sequence. Plasmid DNA was concentrated by ethanol precipitation (Sambrook *et al*., 1989) with some modifications. Plasmid (21 μl) was combined with 2.3 μl 3 M sodium acetate and 46.7 μl 100% ethanol, incubated on ice for 30 min, and collected by centrifugation. Precipitated DNA was dissolved in 10 μl of water. Sequencing reactions contained approximately 300 ng of plasmid DNA and 1 μl of either pETUpstream primer or DuetDOWN 1 primer (10 μM). Sequencing was performed in the Keck Center for Conservation and Molecular Genetics following the ABI protocol.

A verified, concentrated HIF-1α/pETDuet recombinant plasmid (3F) was transformed into *E.coli* BL21 competent cells (Invitrogen). BL21 cells (5 μl) were allowed to thaw on ice, after which 1 μl of the plasmid was added. The transformation protocol was as described above except that after the heat shock step, 80 μl of room temperature SOC was added to the tube. One colony grown on a plate overnight was used to make a 5 ml culture (3Fa) in LB. The culture was incubated for 18 h at 37°C with shaking at 220 rpm. The culture was stored in 80% sterile glycerol at -80°C.

Polyhistidine-tagged HIF-1α peptide was expressed following the QIAexpressionist protocol (Qiagen) with modifications. A sample of the frozen 3Fa/BL21(DE3) stock was obtained by scraping and used to inoculate 50 ml of LB and incubated for 18 h at 37°C with

shaking (220 rpm). The 50 ml culture was used to inoculate 1 l LB and incubated at 37°C with shaking (220 rpm) until an OD_{600} of 0.6 was reached. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Gold Biotechnology) was added to the culture (final concentration of 1 mM) to induce HIF-1α peptide expression and the culture was allowed to incubate for 18 h at 37°C with shaking (220 rpm).

E.coli from multiple 1 l cultures were harvested separately by centrifugation at 8,000 *g* for 10 min at 4°C. Cell pellets were resuspended, lysed, and homogenized in glass homogenizers on ice in lysis buffer containing 20 mM Tris pH 8.0, 50 mM NaCl, and 50mM KCl. To each lysate, 50 μl of protease inhibitor (10X) (Roche) and 40 μl of lysozyme (10 mg/ml) (ICN Biomedicals) were added and incubated on ice for 30 min. Lysates were passed through a French pressure cell disrupter (SIM-AMINCO) at 12,000 psi a total of five times, after which they were centrifuged at 13,000 *g* for 20 min at 4°C. Supernatants were saved and pellets resuspended in lysis buffer for protein analysis (see below).

Polyhistidine tagged HIF-1α peptide was purified by Ni-NTA chromatography. The column contained 5 ml of Ni-NTA agarose (Qiagen) equilibrated with lysis buffer. Lysate supernatants from multiple cultures were applied to the column separately and slowly drained. The flow-through fraction was reloaded on the column and drained two more times. Then, the column was washed with one column volume of Buffer A1 (20 mM Tris-HCl pH 8, 50 mM NaCl, 50 mM KCl, 20 mM Imidazole, and 5% glycerol), then Buffer B (20 mM Tris-HCl pH 8, 500 mM NaCl, and 500 mM KCl), then Buffer A2 (20 mM Tris-HCl pH 8, 50 mM NaCl, 50 mM KCl, and 30 mM Imidazole). The polyhistidine tagged HIF-1α peptide was eluted with 8 x 5 ml washes with Buffer C (20 mM Tris-HCl pH 8, 50 mM NaCl, 50 mM KCl, and 200 mM Imidazole).

Elution fractions from Ni-NTA chromatography were dialyzed against Buffer I (20mM Imidazole, 20 mM NaCl, 1 mM DTT, and 5 mM β-mercaptoethanol) for 18 h at 4°C using Spectra/Por membrane tubing (10,000 molecular weight cut-off; Spectrum Labs), pooled, and loaded on an anion exchange column. The 2.5 x 10 cm column contained 10 ml DEAE Sephacel cellulose (100 μl particle size) (Pharmacia Fine Chemicals) equilibrated with Buffer I. The column was washed extensively with Buffer I and polyhistidine tagged HIF-1α peptide was eluted in 4 x 5 ml fractions of Buffer I containing 250 mM NaCl followed by an additional 4 x 5 ml fractions of Buffer I containing 500 mM NaCl. All fractions from the column were collected for gel electrophoresis and protein determination (see below).

Fractions from anion exchange containing polyhistidine tagged HIF-1α peptide were further purified by size exclusion chromatography. A 1 cm x 30 cm column containing 20 ml of Superdex 75 agarose (GE) was washed with water (to remove ethanol) and equilibrated with Buffer I. Fractions 1 – 3 from the 250 mM NaCl elution of anion exchange were pooled (15 ml) and concentrated to approximately 1 ml using centrifugal concentrators (Amicon Ultra 4, 10 K cutoff). The 1 ml sample was divided into two 500 μl aliquots, which were each concentrated to 50 - 60μl (Amicon Ultra 0.5 ml, 10 K cutoff). These samples were separately applied to the gel filtration column at a flow rate of 0.227 ml/min. The column was washed Buffer I and fractions of approximately 1.1 ml were collected. All fractions from the column were collected for gel electrophoresis and protein determination (see below).

2.3 Protein Assay, Electrophoresis, and Western Blotting

Total protein was determined by the bicinchoninic acid protein assay (Pierce, ThermoFisher Scientific) with bovine serum albumin standards (Smith *et al.*, 1985). Prior to assay, interfering substances were removed by precipitation with trichloroacetic acid and

deoxycholate (Brown *et al.*, 1989). Standards and samples were assayed in triplicate and absorbance was measured with a Beckman DU-640 spectrophotometer.

Polyacrylamide gel electrophoresis (PAGE) employed Bis-Tris NuPage gels (Invitrogen). Protein samples were combined with loading sample buffer (LSB) and 50 mM DTT and heated at 70°C for 10 min. Proteins were routinely separated on 4-12% Bis-Tris NuPage gels in 1X MOPS SDS running buffer (50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7). Prestained molecular weight markers (Invitrogen) and biotinylated molecular weight markers (Cell Signaling) were included for protein visualization in protein staining and western blotting, respectively. Gels ran at 150 V for 65 min at room temperature.

Gels were stained for protein using colloidal Coomassie blue. Gels were fixed for 1 h in 50% (v/v) ethanol and 3% (v/v) phosphoric acid, washed with water for 5 min, and then stained overnight in colloidal Coomassie blue solution (34% v/v methanol, 6% w/v ammonium sulfate, 2% v/v phosphoric acid, and 0.1% w/v Coomassie brilliant blue G-250). Gels were destained with water for up to 3 days. Gel images were captured with a Bio-Rad Chemidoc-XRS imager and analyzed with Quantity One software (Bio-Rad).

For western blotting, gels were transferred to polyvinyldene difluoride (PVDF) membranes for 2 h at 100 V and 10°C using 1X transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA pH 7.2) containing 20% (v/v) methanol and 0.05% (w/v) SDS. After transfer, blots were blocked by incubating in TBS-T (20 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat dry milk powder for 1 h at room temperature. To follow the purification of the polyhistidine-tagged HIF-1α peptide, the primary antibody was the chicken polyclonal IgY directed against *F. heteroclitus* HIF-1α. In other experiments, affinity purified HIF-1α antibodies were used as described below. The primary antibody was diluted 1:500 in TBS-T

containing 5% non-fat dry milk and membranes were incubated for 1 h at room temperature on an orbital shaker, then overnight at 4°C without shaking. After 3 x 5-min washes in TBS-T, donkey anti-chicken antibody conjugated with horseradish peroxidase (Thermo Scientific) diluted to 1:5000 in TBS-T containing 5% non-fat dry milk was added, and blots were incubated for 1 h at room temperature on an orbital shaker. Membranes were then washed 5 x 5-min in TBS-T. The antigen was visualized by incubating membranes in enhanced chemiluminescence detection reagents (an equal mix of 100 mM Tris, pH 8.5, 2.5 mM luminol, 0.4 mM p-coumaric acid and 100 mM Tris, pH 8.5 containing 0.02% H_2O_2) for 1 min. Images were obtained with ChemiDoc XRS and analyzed with Quantity One (Bio-Rad).

In some cases membranes were stripped by incubating in 62.5 mM Tris, pH 6.8, 2% SDS, 114 mM β-mercaptoethanol at 50°C for 30 min, followed by rinsing in running water for 10 min, washing 4 x 5 min in TBS-T, and reblocking in TBS-T containing 5% non-fat dry milk powder for 1 h at room temperature. Stripped membranes were then re-probed with another antibody.

2.4 Destaining and Digestion of Gel Slices for Mass Spectrometry

Mass spectrometry (MS) was used to identify proteins in two experiments: the purification of polyhistidine-tagged HIF-1α peptide and to verify that affinity purified HIF-1α antibodies recognize full-length HIF-1α from killifish cells (see below). For the first application, all steps up to MS were done at the University of New Orleans and the MS analysis was carried out by Dr. Yang Cai at the Research Institute for Children, Children's Hospital New Orleans as described in Abbaraju *et al.* (2011). For the second application, slices of polyacrylamide gels were sent for digestion and MS analysis by the Proteomics Core at the University of British Columbia, Canada. Steps performed in this thesis for the first application are described below.

Coomassie-stained gel bands (approximately 10 mm x 3 mm x 1 mm) corresponding to polyhistidine-tagged HIF-1α peptide were excised from polyacrylamide gels and diced with a clean scalpel (ca. 3 mm x 3 mm x 1 mm). Gel fragments were washed with 500 μl water, then incubated sequentially in 200 μl of the following solutions each for 10 min with frequent vortex mixing: 50 mM ammonium bicarbonate (NH_4HCO_3); a 1:1 mixture of acetonitrile and 50 mM NH_4HCO_3 solution; acetonitrile. Incubation steps were repeated, discarding the solutions each time, until all Coomassie blue was removed. Gel fragments then were submerged in 100 μl 10 mM DTT in 50 mM NH_4HCO_3 and incubated for 1 h at 45 °C. After cooling to room temperature, the solution was removed and replaced with acetonitrile and incubated for 10 min. The acetonitrile was removed and replaced by 100 μl of 55 mM iodoacetamide in 50 mM NH_4HCO_3 and incubated for 45 min in the dark with occasional vortexing. The solution was removed and gel fragments were incubated in 200 μl 50 mM NH_4HCO_3 for 10 min. The solution was removed and gel fragments were dehydrated by adding 200 μl acetonitrile for 10 min. The liquid was removed and gel fragments were dried in a speedvac for 15 min on aqueous setting. The gel fragments were rehydrated in 20 μl of 50 mM NH_4HCO_3 containing 200 ng trypsin and placed on ice for 20 min. Without removing the solution, another 20 μl of 50 mM NH_4HCO_3 without trypsin was added and incubation was continued for 18 h at 37°C. Another 40 μl of water was added and the incubation was continued another 30 min at 37°C. The liquid was removed and saved in a clean microcentrifuge tube. Then, gel fragments were incubated sequentially in 50 μl of each of the following solutions each for 10 min, vortexing at 5 min, and removing and saving liquid each time into the same tube: acetonitrile; 25 mM NH_4HCO_3; acetonitrile. Then 50 μl 25 mM NH_4HCO_3 was added, fragments incubated 10 min, with vortexing at 5 min. Without removing the liquid, 50 μl acetonitrile was added, fragments

incubated 10 min, with vortexing at 5 min. The liquid was removed and pooled with the volumes collected earlier. These last washes with 25 mM NH_4HCO_3 and acetonitrile were repeated, collecting and pooling liquid. Finally, 50 μl acetonitrile was added, fragments were incubated 10 min, with vortexing at 5 min, and this liquid was saved and pooled with the other volumes reserved from the same sample. The combined supernatants, containing digestion products from individual gel bands, were dried in a speedvac for 2.5-3 h on aqueous setting. Samples were saved at −20°C until MS analysis.

2.5 Affinity Purification of HIF-1α Antibodies

Fractions from gel filtration chromatography (Section 2.2) containing polyhistidine-tagged HIF-1α peptide were pooled. The buffer was replaced with Coupling Buffer (0.1 M sodium citrate and 0.05 M sodium carbonate, pH 10) by centrifugation at 4,000 *g* for 10 min, 4°C, using Amicon Ultra 4 device (EMD Millipore). A total of 2.5 mg of purified polyhistidine-tagged HIF-1α peptide was coupled to 2 ml of AminoLink Plus Resin (Thermo Scientific) following the manufacturer's protocol. This column, in turn, was used to purify anti HIF-1α antibodies from chicken polyclonal IgYs (Section 2.1). The affinity purification followed the manufacturer's protocol with the antibodies finally eluted with 0.1 M glycine, pH 2.5, that was immediately neutralized by the addition of $1/10^{th}$ the fraction volume of 1 M Tris, pH 8.

Dot blotting of column fractions was used to evaluate the presence of anti HIF-1α antibodies. Using a Bio-Rad dot blot apparatus, a 0.45 μm nitrocellulose membrane (Bio-Rad) was first exposed to polyhistidine-tagged HIF-1α peptide. To do this, 0.24 mg semi-purified polyhistidine-tagged HIF-1α peptide (gel filtration fractions immediately before or after peak HIF-1α peptide concentration) was diluted in 10 ml TBS, and 100 μl of this mixture (equivalent to 2.4 μg protein) was applied to each of 96 wells for 30 min. After 30 min, excess solution was

removed by vacuum, and the membrane was blocked in TBS-T for 30 min. Excess TBS-T was removed by vacuum, and serial dilutions of affinity column fractions were applied to the membrane for 30 min. Excess solution was removed by filtration, and the membrane was washed with TBS-T. The dot blot apparatus was disassembled, and membranes were blocked overnight at 4°C in TBS-T with 5% non-fat milk powder. Membranes were washed 3 x 5-min in TBS-T then incubated for 1 h at room temperature with HRP-conjugated donkey anti-chicken IgG (Thermo Scientific) diluted 1:5000 in TBT-T containing 5% non-fat milk. Membranes were then washed 3 x 5-min in TBS-T and 1 x 5 min in TBS, developed for chemiluminescence, and imaged as described above (section 2.3). Elution fractions with the highest reactivity were pooled, dialyzed against TBS with 0.1% sodium azide overnight, the stored at −20°C.

2.6 Immunoprecipitation

The TNT Coupled Reticulocyte Lysate System (Promega L4611) was used to generate full length HIF-1α, 2α, 3α, and ARNT as controls for western blotting and immunoprecipitation. *In vitro* transcription and translation (IVTT) was carried out according to the manufacturer's protocol using pcDNA vectors encoding the appropriate cDNA from *F. heteroclitus* (Townley *et al*., in prep) with the addition of 200 μg/ml MG132 to inhibit endogenous proteosomal activity. Completed reactions were aliquoted and frozen at −80°C until used.

The basic protocol for immunoprecipitation (IP) followed the protocol from Aves Labs. The standard conditions are described below, with specific conditions given in the Results section. Immunoprecipitation buffer (IP buffer) was 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.76 mM NaH_2PO_4 (final pH 7.4 – 7.5), 1% (v/v) Igepal, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM sodium orthovanadate, 1% (v/v) protease inhibitor cocktail (Sigma P8340) and 50 μg/ml MG 132. During optimization of IP, IVTT HIF-1α (1 – 10

μl) was added to 1.0 ml IP buffer at 4°C. To every IP reaction, affinity-purified HIF-1α antibody (4 μl, equivalent to 4 μg IgY) was added and reactions were incubated on ice (1 or 2 h depending upon experiment), inverting tubes every 30 min. PrecipHen reagent (agarose-coupled goat anti-chicken IgY; Aves Lab; 16 – 40 μl depending upon experiment) was added to each reaction and incubated at 4°C with end-over-end rocking (for 3 h or overnight depending upon experiment). Samples were centrifuged at 1,500 *g* for 5 min, and supernatants, which contained unbound antigen or antigen-antibody complexes not precipitated by PrecipHen reagent, were saved (hereafter referred to as "supernatants"). Precipitated antigen-antibody-PrecipHen complexes were washed sequentially in 0.5 ml each of TBS-T, TBS, and 50 mM Tris, pH 6.8, centrifuging each time at 1,500 *g* for 5 min. The final pellets were resuspended in 40 μl 1X LSB containing 50 mM DTT and heated at 70°C for 10 min. After heating, samples were centrifuged at 15,000 *g* for 5 min, and supernatants, which contained immunoprecipitated antigen, were saved (hereafter referred to as "pellets"). In some cases, the first supernatants were concentrated using centrifugal concentrators or analyzed without concentration by combining 15.6 μl with 8 μl 4X LSB and 2.4 μl 500 mM DTT and heated as above. All samples were analyzed by western blotting as described earlier, except using the affinity-purified anti-HIF-1α antibody.

2.7 Cell Culture Experiments

Several experiments used killifish embryo-5 (KFE-5) culture cells, a continuous cells line of myogenic origin (Gignac *et al*., 2014). The culturing of the cells and preparation of cell lystates, cytosolic extracts, and nuclear extracts were kindly performed by Mr. Dillon Chung working in Dr. Patricia Schulte's laboratory at the University of British Columbia. All analyses of total protein and HIF-1α were done at the University of New Orleans as part of this thesis.

KFE-5 cells were maintained at 21-25°C in T-75 tissue culture flasks on a growth medium of Leibovitz's L-15 supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin. Routinely, experiments were started by seeding multiple replicate T-75 flasks at 1.85 x 10^5 cells each (determined using a standard hemocytometer). All experiments were conducted in normoxic conditions. In some cases, $CoCl_2$ was included as a hypoxic mimetic (Co^{2+} inhibits prolyl hydroxylation) at concentrations and periods of time shown in the Results. At the desired time, and prior to reaching confluency, cells were detached using commercially available TrypLE and washed 3 times using ice cold PBS (137mM NaCl, 2.7mM KCl, 10.1mM Na_2HPO_4, 1.76mM KH_2PO_4, pH 7.6), collecting cells by centrifugation at 600 *g* for 10 min at 4°C between washes. After the final wash, the supernatant was removed and packed cells were lysed by the addition of ice-cold IP buffer, followed by vortex mixing. Extracts were centrifuged and supernatants were snap frozen in liquid N_2 and stored at −80°C.

In one experiment, cytosolic and nuclear fractions were separated. After the final wash of cells in PBS, the supernatant was removed and packed cells were lysed in 500 µl of Buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% Igepal, 50 µM MG-132 and 1% Sigma protease inhibitor cocktail, pH7.9) by repeated pipetting and vortexing (15 s). After incubating for 10 min on ice, extracts were centrifuged at 6,000 *g* for 10 min at 4°C. The supernatants (cytosolic extract) were removed, snap frozen in liquid N_2, and stored at −80°C. Nuclear pellets were resuspended in 200 µl of Buffer B (420 mM NaCl, 5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), pH7.9) and pooled. Nuclear suspensions were vortexed for 15 s and incubated for 30 min on ice with 15 s of vortexing every 10 min. This suspension was centrifuged at 24,000 *g* for 20 min at 4°C. The supernatant was kept as the nuclear extract, snap frozen in liquid N_2, and stored at −80°C.

Prior to IP, cytosolic and nuclear fractions were thawed and dialyzed against IP buffer. Immunoprecipitation followed the general protocol described above with the following modifications. The desired amount of lysate protein (from 0.6 to 2.2 mg, depending upon experiment) was brought to a total volume of 1.0 ml with IP buffer. A "pre-clearing" step was added to remove lysate proteins that bind non-specifically to the PrecipHen reagent. For this, 20 μl of PrecipHen was added to each reaction and incubated for 30 min at 4°C with end-over-end rocking. Reactions were centrifuged at 1,500 *g* for 10 min at 4°C. The pellets were treated as described above for western blotting (hereafter referred to as "pre-clear"). The supernatant from this step served as the starting material for IP as described above (i.e., starting with the addition of anti-HIF-1α antibody).

From two experiments with KFE-5 cells, bands in Coomassie-stained protein gels corresponding to the molecular weight for HIF-1α (determined from western blotting the same samples in parallel gels) were excised for MS analysis. Gel destaining, protein digestion, and tandem MS analysis were performed by the Proteomics Core at the University of British Columbia, Canada.

2.8 Animal Exposures and Tissue Lysates

Fundulus grandis were purchased from Joe's Landing (Barrataria, LA, USA) and kept at room temperature (19-24°C) in dechlorinated water brought to a salinity of 10 – 13 ppt with Instant Ocean Synthetic Sea Salt. The fish were acclimated for a minimum of 2 weeks to laboratory conditions, during which time they were fed 5 to 7 times a week with TetraMin Tropical Flake food. Partial water changes were performed every 2 weeks. The fish were exposed to hypoxia by gassing the aquarium water with a mixture of nitrogen and air from cylinders of compressed gasses. The dissolved oxygen concentration was measured with a YSI

oxygen-temperature-salinity probe (Yellow Springs Instruments Model Pro2030). The level and duration of hypoxia are given in the Results section. All experiments with live animals were performed according to guidelines for research on vertebrate animals (UNO IACUC Protocols 14-003 and 15-004).

At the end of the hypoxia exposure, fish were euthanized with tricaine methanesulfonate (MS 222), and tissues (skeletal muscle, liver, gonad, and gill) were dissected, frozen in liquid nitrogen, and stored at −80°C. Frozen fish tissues were pulverized under liquid nitrogen in a pre-cooled mortar and pestle. Powdered tissues were homogenized in ice-cold IP buffer with 10 strokes of a Teflon pestle in a glass tissue homogenizer (size BB; Thomas Scientific). Lysates were centrifuged at 10,000 *g* for 10 min at 4°C. Supernatants were removed and saved at −80°C until analyzed by IP as described above including the pre-clearing step.

3. RESULTS

3.1 Cloning, Expression, and Purification of Polyhistidine-tagged HIF-1α Peptide

A partial cDNA of *F. heteroclitus* HIF-1α was successfully subcloned into the pETDuet expression vector. Sequencing of the insert showed >99% identity with the expected region of *F. heteroclitus* HIF-1α (Figure 3). The predicted molecular mass of the polyhistidine-tagged HIF-1α peptide, including amino acids encoded by the vector, was 13.8 kD. During purification of proteins expressed by *E. coli* transformed with the HIF-1α/pETDuet vector, a prominent band at approximately 16 kD was observed. When *E. coli* extracts were subjected to chromatography on Ni-NTA and eluted with 200 mM imidazole, this band was greatly enriched (Figure 4A), suggesting that it was the recombinant HIF-1α peptide. At this concentration of imidazole, a small number of higher molecular weight proteins co-purified with the polyhistidine-tagged HIF-1α peptide. Trials of Ni-NTA chromatography using a step gradient of imidazole (50, 100,150, and 200 mM) did not result in any preferential enrichment of the 16 kD protein. Therefore, proteins eluted from Ni-NTA chromatography were further fractionated by anion exchange on DEAE-cellulose. Bound proteins were eluted at 250 mM NaCl, followed by 500 mM NaCl. The 16 kD band eluted at the lower NaCl concentration. Several bands co-purified under these conditions (Figure 4B). Although the 16 kD band was expected to be the polyhistidine-tagged HIF-1α peptide, this was verified by MS analysis of several bands excised from peak fractions at 250 mM and 500 mM NaCl (Figure 4C). The 16 kD band (band 1) was identified as *F. heteroclitus* HIF-1α, with a p value of 2.20×10^{-15}. No other bands matched *F. heteroclitus* HIF-1α (Table 1).

The expression and purification of polyhistidine-tagged HIF-1α peptide through anion exchange was repeated with multiple 1-liter cultures of *E. coli* BL21(DE3) transformed with

plasmid 3Fa (Figure 5A, 5B). Peak fractions from anion exchange (250 mM NaCl, fractions 1-3) were combined, concentrated, dialyzed, and further purified by gel filtration chromatography, allowing the polyhistidine-tagged HIF-1α peptide to be separated from higher molecular weight contaminants (Figure 5C). Figure 6 shows the overall purification of the polyhistidine-tagged HIF-1α peptide. With every chromatography step, the number of contaminants was reduced, resulting in highly purified polyhistidine-tagged HIF-1α peptide. The yield of the polyhistidine-tagged HIF-1α peptide when starting with 4 x 1-l cultures was 2.56 mg protein (Table 2).

3.2 Affinity Purification of HIF-1α Antibodies

A total of 2.5 mg of purified polyhistidine-tagged HIF-1α protein was used to make a 2 ml affinity column (AminoLink). A representative dot blot of fractions (2 ml each) from one round of purification is shown in Figure 7. The starting material (purified IgY fraction) has high reactivity (left lane). The flow through showed very little reactivity—the dot intensity at 1:100 dilution is approximately the same as the dot intensity of the 1:12,800 dilution of the starting material. The sixth fraction began to show more reactivity, indicating that the capacity of the column had been exceeded after 10 ml of IgY had been applied to the column. After washing, the reactivity against HIF-1α was recovered by eluting bound antibody with three column volumes of 0.1 M glycine, pH 2.5. A total of 731 mg of IgY from hen #6210 in 32 ml was applied to the affinity column in three rounds of chromatography. The total yield of purified HIF-1α antibody was 12.4 mg in 13.5 ml of eluate (0.9 mg/ml). The HIF-1α antibody was less than 2% of the total IgY protein, which is consistent with the expected abundance of specific antibodies against an antigen of interest (Aves Labs).

Western blot analysis of IVTT full length *F. heteroclitus* HIF-1α, 2α, 3α and ARNT was used to determine the specificity of the unpurified IgY and the affinity-purified HIF-α antibody

(Figure 8). The major band recognized by both the unpurified and the purified antibodies is HIF-1α (ca. 100 kD band in lanes labeled 1α). Both unpurified and purified antibodies cross-react with other abundant proteins in the IVTT reactions. But the affinity-purified antibodies showed fewer cross-reactive low molecular weight bands (MW < 60 kD) and essentially no cross-reactivity against IVTT HIF-2α, HIF-3α, or ARNT (1β).

3.3 Immunoprecipitation Optimization

Preliminary attempts to detect HIF-1α by western blotting of total tissue lysates did not show a reproducible band of the correct molecular weight, presumably because, as a transcription factor, the abundance of HIF-1α is very low. Thus, IP was optimized as an approach to enrich and measure HIF-1α levels. To start, the concentration of antibody, the concentration of precipitating reagent (PrecipHen), and the incubation times were optimized for IP of IVTT HIF-1α added to IP buffer. The binding capacity of 1 ml PrecipHen (packed volume) is 1 mg chicken IgY and the recommended ratio of PrecipHen to antibody is 2:1 (Aves Labs). PrecipHen is provided as a hydrated resin and 2 ml hydrated resin is equivalent to 1 ml packed volume. So, the ratio of hydrated PrecipHen to precipitating antibody should be no less than 4 ml:1 mg. At a constant amount of IVTT HIF-1α (10 μl in 1.0 ml IP reactions), the total amount of antibody and PrecipHen were increased while maintaining this 4:1 ratio (Figure 9A). Every reaction showed that some IVTT HIF-1α remained in the supernatant, suggesting that either the antibody or PrecipHen reagent was not sufficient. Because the band intensity of HIF-1α in the pellet did not increase with increasing volume of antibody (Figure 9A), this suggested that the PrecipHen reagent, not the primary antibody was limiting. For the remainder of the experiments, 4 μl (ca. 4 μg) of affinity-purified HIF-1α antibody was used in each IP reaction.

To determine the optimal ratio of PrecipHen to antibody, increasing volumes of PrecipHen were included in IP reactions using 4 μl (ca. 4 μg) of affinity-purified HIF-1α antibody (Figure 9B). IVTT HIF-1α was cleared from the supernatant at >16 μl PrecipHen. For the remainder of the experiments, a volume of 20 μl of PrecipHen (10 μl packed volume) was used in each IP reaction. This experiment also included control reactions with no IVTT lysate or IVTT lysate that had no pcDNA to evaluate non-specific binding of rabbit reticulocyte proteins by PrecipHen reagent (none observed).

Next, optimal incubation times for the primary antibody and the precipitating reagent were determined (Figure 9C). Incubation times of 1 or 2 h with the affinity-purified HIF-1α antibody were equivalent in clearing the supernatant of IVTT HIF-1α. For the precipitating reagent (PrecipHen), however, 3 h incubation was not long enough, as shown by the substantial amounts of IVTT HIF-1α remaining in the supernatant. Overnight incubation with PrecipHen, on the other hand, effectively cleared the supernatant of IVTT HIF-1α. Therefore, an incubation of 1 h with primary antibody followed by overnight incubation with PrecipHen was used for the remainder of the experiments. To evaluate the ability of the affinity-purified HIF-1α antibody to precipitate lower amounts of IVTT HIF-1α, the volume of IVTT added to IP buffer was decreased from 10 μl to 1 μl (Figure 9D). The antibody precipitated IVTT HIF-1α down to 1 μl. Lower amounts of IVTT HIF-1α were not tested.

3.4 Immunoprecipitation of HIF-1α from KFE-5 Cell Extracts

The optimized IP protocol was next applied to extracts derived from the *F. heteroclitus* cell line, KFE-5. The initial experiments were done with total cell lysates prepared from KFE-5 cells grown under normoxic conditions (no $CoCl_2$). Increasing amounts of IVTT HIF-1α were added to aliquots of identical KFE-5 extracts prior to IP. A pre-clearing step was included to

remove proteins that bind non-specifically to the PrecipHen reagent (see Methods). With increasing volume of IVTT HIF-α added, a band of increasing intensity was observed at 100 kD in the final pellets, whereas no band of the same molecular weight was observed in the pre-clearing step or in the initial IP supernatant. Thus, the optimized protocol effectively precipitates added IVTT across this range. Importantly, an endogenous band of the same molecular weight was observed in KFE-5 lysates with no added IVTT HIF-1α (Pellet, lane 0) (Figure 10). This experiment not only validated the IP protocol for cell lysates, but suggest that normoxic KFE-5 cells express HIF-1α.

Next, KFE-5 cells were incubated with 0 and 100 μM $CoCl_2$ for 24 h to see if the intensity of this 100 kD protein increases during exposure to this hypoxic mimetic. Total cell lysates were used in IP with the affinity-purified antibody. There was variation in the intensity of the 100 kD band among replicate lysates (representing replicate cell culture flasks) with or without $CoCl_2$ (Figure 11A). One $CoCl_2$ lysate (sample 2) showed an aberrant banding pattern, presumably due to protein degradation, making quantitative comparison difficult. Overall, there was no apparent increase in band intensity in the presence of $CoCl_2$

The next experiment varied the concentration of $CoCl_2$ and the duration of exposure. Again, there was no obvious effect $CoCl_2$ on the intensity of the 100 kD band (Figure 11B). All three lysates from cells exposed to 100 μM $CoCl_2$ showed low intensities for the 100 kD band, as well as for the immunoprecipitating IgY band (65 kD), suggesting some sample loss occurred during IP. Therefore, the IP of the 0 μM and 100 μM $CoCl_2$ were repeated. In this experiment, the 6 and 24 h samples from 0 μM $CoCl_2$ were pooled and compared to the 24 h sample from 100 μM $CoCl_2$, enabling an increase in the total protein used in IP to 2.15 mg (Figure 11C).

Densitometry and image analysis using Quantity One (BioRad) revealed a small increase in intensity (20-30%) of the 100 kD band in the $CoCl_2$ treated sample.

To learn if the protein at 100 kD translocates from the cytosol to the nucleus during $CoCl_2$ exposure, KFE-5 cells were incubated with 0 and 100 μM $CoCl_2$ for 24 h. Cell extracts were separated into cytosolic and nuclear fractions, and IP was carried out on the entire protein in cytosolic and nuclear fractions pooled from several cell culture flasks. After IP, increasing volumes of the final precipitated material from each fraction were used for western blotting (Figure 12). By comparing lanes containing the same volume of IP material (e.g., 6 μl) from cytosolic and nuclear fractions, it is clear that staining intensity was more intense for nuclear extracts. Alternatively, if one compares lanes of similar intensity and asks what volume of IP material was used, then similar band intensities were observed when half as much nuclear extract was electrophoresed compared to cytosolic extracts (e.g., compare 6 μl of nuclear extract to 12 μl of cytosolic extract). The trend towards greater staining intensity in nuclear extracts was the same regardless of $CoCl_2$, suggesting that nuclear localization of 100 kD protein was unaffected by this hypoxic mimetic. Image analysis using the intensity of the IgY band to control for efficiency of the IP, verified that 100 kD protein is 2-fold enriched in the nucleus compared to the cytosol, both with and without $CoCl_2$.

The same samples from IP of cytosolic and nuclear fractions of KFE-5 cells exposed for 24 h to 0 and 100 μM $CoCl_2$ were electrophoresed and stained with colloidal Coomassie blue. Two bands between 70 and 120 kD were barely visible (Figure 13). Based upon the sensitivity of colloidal Coomassie blue (Candiano *et al*., 2004), the protein content of these bands was on the order of 50-100 ng. Two gel regions from IP of nuclear extracts, corresponding to roughly 90 to 120 kD (bands 1 and 3; Figure 13B) and 70 to 90 kD (bands 2 and 4) were excised for MS

analysis. Prior to in-gel digestion and MS analysis, gel bands of a given MW range from cells exposed to 0 and 100 μM $CoCl_2$ were pooled. In a parallel gel used in western blotting, the affinity-purified HIF-1α antibody showed strong reaction with a band in the heavier MW range (ca 100 kD). Tandem MS analysis followed by automated database searching matched 4 peptides from gel bands 1+3 to HIF-1α from the Southern platyfish (*Xiphophorus maculatus*)(combined score = 74). Subsequent manual analysis showed the same peptides exist in *F. heteroclitus*. Analysis of bands 2+4 did not provide matches with any HIF-α subunits.

3.5 Immunoprecipitation of HIF-1α from *F. grandis* tissues

To reveal if the affinity-purified HIF-1α antibodies precipitate HIF-1α protein from tissue samples, extracts of several tissues of the Gulf killifish, *F. grandis*, were prepared and analyzed by IP. To begin, IVTT full length HIF-1α was added to tissue lysates from normoxic fish to determine if anything in the extract interfered with the IP. As with KFE-5 cell extracts, the pre-clearing step was included in the IP protocol. The IVTT HIF-1α was immunoprecipitated, but with variable degrees of efficiency: there was better recovery from extracts of skeletal muscle and ovary than from extracts of liver and gill (Figures 14A-D). For comparison, the amount of IVTT that was added in the 10 μl volume should have produced a band intensity (if 100% recovered) equivalent to the band intensity shown in the input lane (far left). The low recoveries of added IVTT HIF-1α in liver and gill could be the result of higher protein degradation in these extracts despite the inclusion of protease inhibitors in all tissue lysates. Similar to experiments with KFE-5 cells, affinity-purified antibody recognized a protein at approximately the same molecular weight as the full-length HIF-1α (100 kD) in muscle, liver, and ovary. Based upon similarity of molecular weight, and given the positive identification in KFE-5 cells, it is likely that this protein is *F. grandis* HIF-1α.

Two experiments were done to investigate whether hypoxic exposure of live fish affects the amount of HIF-1α protein. In the first experiment, three fish were used: one was sampled after being held under normoxic conditions (0 h hypoxia), and one each was sampled after 24 h and 96 h at approximately 0.9 mg/l dissolved oxygen (11% of the air saturated value). Tissues were collected, lysates prepared, and IP conducted. Pellets from IP showed a very faint band in all tissues at 100 kD, which appeared darker in one or both hypoxic samples (24 h and 96 h) compared to the normoxic sample (0 h) in all tissues. Due to the low band intensity, this experiment was repeated with four fish, two held under normoxia and two held under hypoxia (0.6 mg/l oxygen or 7.5% air saturation) for 24 h. In this experiment, gill was not analyzed because of the low recoveries of protein in IP. The supernatants of IP reactions, representing the bulk of the lysate proteins, were electrophoresed and stained for total protein (Figure 16A). This showed that, for a given tissue, approximately equal amounts of protein were used in the IP reactions (corroborating the protein assay). The western blot of the IP is shown in Figure 16B. A protein of 100 kD was recovered in extracts made from tissues of hypoxic fish. The same protein was either absent (muscle) or present at lower intensities (ovary and liver) in samples prepared from normoxic fish. This result strongly supports the specificity of the affinity-purified antibody for HIF-1α, and it is also one of the only reports of hypoxic induction of HIF-1α protein after short term exposure of live fish to low oxygen.

4. DISCUSSION

Many aquatic habitats are characterized by variable concentrations of dissolved oxygen. Fish that occupy these habitats respond to changes in oxygen levels through behavioral, physiological, and biochemical adjustments. Past studies suggest the transcription factor HIF-1α has an important role in the molecular responses to hypoxia in fish (Nikinmaa and Rees, 2005; Richards, 2009). The cDNA sequence and mRNA abundance HIF-1α have been reported for many species. Less is known about HIF-1α protein levels, primarily due to a lack of tools to effectively detect the protein from fish, specifically antibodies that recognize fish HIF-1α. Because post-transcriptional control of HIF-1α is the major determinant of protein abundance and function, at least in mammals, better information on HIF-1α protein levels is critical to understanding the function of this transcription factor in the hypoxia responses of fish.

Previously, only a few studies have used antibodies to evaluate HIF-α protein in fish. Soitamo *et al*. (2001) developed a polyclonal antibody against rainbow trout HIF-1α. The antibody was purified by Ni^{2+} and gel filtration chromatography. Western blotting of nuclear extracts of rainbow trout gonad cells (RTG-2) and Chinook salmon embryonic cells (CHSE-214) showed that HIF-1α protein was present under conditions of moderate hypoxia, peaking at 5% O_2 (equivalent to about 25% of the air-saturated value or a DO of 1.9 mg/l). This level of oxygen is similar to that which prevails in the capillaries under normoxic conditions. In both cell lines, HIF-1α protein levels decrease as oxygen was dropped to lower values (Soitamo *et al*., 2001). Sollid *et al.* (2006) utilized the same antibodies against rainbow trout HIF-1α to characterize HIF-1α in crucian carp. The N-terminus of HIF-1α (from which the antibodies were developed) is similar between the crucian carp and rainbow trout. The antibodies were added to reaction mixture containing crucian carp nuclear protein. An EMSA was utilized to reveal the binding of

crucian carp HIF-1α to hypoxia response elements in human EPO gene. In the same study, HIF-1α protein accumulation was observed by Western blot of nuclear proteins from crucian carp gills after a 6 h hypoxia exposure, with levels decreasing to normoxic levels after a 48 h hypoxia exposure (Sollid *et al.* 2006). Lower amounts of HIF-1α protein were also present under normoxic conditions and these were related to body mass. Thomas and Rahman (2009) tested the effects of hypoxia exposure in Atlantic croaker. HIF-1α and HIF-2α protein expression was measured after 2 and 4 weeks (2α only) hypoxia exposure at 4.5% O_2 (DO 1.7 mg/l). Nuclear extracts from croaker ovarian samples were analyzed by western blot using commercially available rabbit polyclonal antibodies against human HIF-1α and HIF-2α. After the continuous hypoxia exposure, levels of both HIF-1α and HIF-2α protein increased in ovaries. Compared to normoxic samples, 2 week hypoxic samples showed the concentration of HIF-1α protein to be 3.0 fold higher (Thomas and Rahman, 2009). Additionally, it was observed that HIF-2α protein levels increased more slowly but were significantly higher than control values under those DO conditions after 4 weeks. Zhang *et al.* (2012) reported on the less understood HIF-3α. The group developed their own polyclonal antibodies raised in rabbits against zebrafish HIF-3α to investigate the effect of hypoxia on HIF-3α protein abundance. Western blotting was used to determine the specificity of the antibodies utilizing HEK293T (human embryonic kidney cells) transfected with GFP tagged zebrafish HIF-1α and HIF-3α plasmids. Endogenous HIF-3α protein was observed in both embryos and adult fish by Western blot after a 12 h hypoxia exposure at 9 % air saturation (DO 0.6 mg/l) for embryos and 26 % air saturation (DO 1.5 mg/l) for adults.

The overall goal of this thesis was to purify and validate antibodies that could be used in various applications to measure HIF-1α protein in *Fundulus heteroclitus* and its sister species *F.*

grandis. Both are widely dispersed salt marsh fish that serve as model organisms in studies of environmental biology due to their high tolerance of stresses such as changes in temperature, salinity, and oxygen concentrations (Burnett *et al*., 2007; Brown *et al*., 2011). They are among the more hypoxia tolerant fishes in the salt marsh habitat; the *F. heteroclitus* genome has been sequenced; there is a *F. heteroclitus* cultured cell line; and both species are easy to collect and maintain in the lab. These features made *F. heteroclitus* and *F. grandis* ideal for this study.

The first objective of this thesis was to purify polyclonal antibodies against the *F.heteroclitus* HIF-1α protein subunit. Since polyclonal antibodies contain the whole inventory of antibodies circulating in the immunized animal, they exhibit more versatility than monoclonal antibodies. However, a crude IgY fraction generates nonspecific staining in a western blot (Camenisch *et al*., 1999; Hill, 2012). By purifying the polyclonal antibodies against the protein of interest, nonspecific staining is avoided (Camenisch *et al*., 1999). The first objective of this thesis was accomplished by cloning and expressing a partial cDNA sequence from *F. heteroclitus* HIF-1α and isolating the recombinant protein through a sequence of nickel, ion-exchange, and size exclusion chromatography. The recombinant HIF-1α protein was then used to affinity purify chicken polyclonal antibodies specific against *F. heteroclitus* HIF-1α protein.

The second objective of this thesis was to measure HIF-1α from killifish cells and tissues. This required that the technique of immunoprecipitation be optimized, first in IP buffer, then in cell and tissue extracts. By adding IVTT HIF-1α to IP buffer and varying the volume of PrecipHen, incubation time for both PrecipHen and affinity-purified HIF-1α antibody, and the volume of IVTT HIF-1α, the optimal conditions for recovery of HIF-1α were achieved. These conditions were then used to immunoprecipitate endogenous HIF-1α protein from the *F. heteroclitus* KFE-5 cell line and from tissue extracts of the closely related *F. grandis*.

Endogenous HIF-1α protein was found in the KFE-5 cells and in the *F. grandis* muscle and gonad. Recovery of HIF-1α protein from *F. grandis* gill and liver was poor, probably because of proteolytic degradation. The affinity-purified HIF-1α antibody also detected a modest increase in levels of HIF-1α protein during $CoCl_2$ incubation of KFE-5 cells and a more robust induction of HIF-1α protein in *F. grandis* tissue after exposure of live animals to low oxygen. These observations extend what has been described in other studies on other fish (Soitamo *et al*., 2001; Sollid *et al*., 2006; Law *et al*., 2006; Thomas and Rahman, 2009; Zhang *et al*., 2012).

In terms of cellular localization, about twice as much HIF-1α protein was found in the nuclear fraction of KFE-5 cells compared to the cytosolic fraction, and this proportion did not differ between normoxic and $CoCl_2$ treated cells. While the $CoCl_2$ response in KFE-5 cells requires further study, the observation of HIF-1α protein during normoxia in these cells and certain tissues from *F. grandis* coincide with other fish studies that show HIF- 1α protein is present during normoxia (Soitamo *et al*., 2001; Rissanen *et al*., 2006; Sollid *et al*., 2006). The presence of HIF-1α during normoxic conditions, suggests a role for oxygen-independent functions (Soitamo *et al*., 2001).

Based on the results presented here, the goals of this thesis to affinity-purify HIF-1α antibodies and use them to detect HIF-1α from in killifish cells and tissues were achieved. These accomplishments confirm the advantages of using affinity-purified IgYs in studies of protein abundance. In addition to immunoprecipitation and western blotting, affinity-purified antibodies can be utilized in affinity chromatography, immunohistochemistry, and chromatin immunoprecipitation. In conclusion, this affinity-purified antibody represents a new tool in studying the mechanisms of hypoxia response in *F. heteroclitus* and *F. grandis*, and by extrapolation in other fish and vertebrates in general.

5. REFERENCES

Abbaraju NV, Cai Y, and Rees BB. 2011. Protein recovery and identification from the gulf killifish, *Fundulus grandis*: comparing snap-frozen and RNAlater preserved tissues. PROTEOMICS. 2011: 4257-4261.

Brown CA, Gothreaux CT, and Green CC. 2011. Effects of temperature and salinity during incubation on hatching and yolk utilization of Gulf killifish *Fundulus grandis* embryos. AQUACULTURE. 315: 335-339.

Brown RE, Jarvis KL, and Hayland KJ.1989. Protein measurement using bicinchoninic acid: elimination of interfering substances. ANAL BIOCHEM. 180: 136-9.

Burnett KG, Bain LJ, Baldwin WS, Callard CV, Cohen S, Di Giulio RT, Evans DH, Gomez-Chiarri M, Hahn ME, Hoover CA, Karchner SI, Katoh F, MacLatchy DL, Marshall WS, Meyer JN, Nacci DE, Oleksiak MF, Rees BB, Singer TD, Stegeman JJ, Towle DW, Van Veld PA, Vogelbein WK, Whitehead A, Winn RN, and Crawford DL. 2007. *Fundulus* as the premier teleost model in environmental biology: Opportunities for new insights using genomics. COMP BIOCHEM PHYS D. 2: 257-286.

Camenisch G, Tini M, Chilov D, Kvietikova I, Srinivas V, Caro J, Spielmann P, Wenger RH, and Gassmann M. 1999. General applicability of chicken egg yolk antibodies: the performance of IgY immunoglobulins raised against the hypoxia-inducible factor 1α. FASEB J. 13: 81-88.

Candiano, G, Bruschi, M, Musante, L, Santucci, AL, Ghiggeri, GM,Carnemolla, B, Orecchia, P, Zardi, L, and Righetti, PG. 2004. Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. ELECTROPHORESIS. 25: 1327-1333.

Cochran RE and Burnett LE. 1996. Respiratory responses of the salt marsh animals, *Fundulus heteroclitus, Leiostomus xanthurus*, and *Palaemonetes pugio* to environmental hypoxia and hypercapnia and to the organophosphate pesticide, azinphosmethyl. J EXP MAR BIOL ECOL. 195: 125-144.

Diaz R and Rosenberg R. 1995. Marine Benthic Hypoxia: A review of its ecological effects and the behavioural responses of benthic macrofauna. OCEANOGR MAR BIOL. 33: 245-303.

Fangue NA, Podrabsky JE, Crawshaw LI, and Schulte PM. 2009. Countergradient variation in temperature preference in populations of killifish *Fundulus heteroclitus*. PHYSIOL BIOCHEM ZOOL. 82: 776-786.

Gassmann M, Thommes P, Weiser T, and Hubscher U. 1990. Efficient production of chicken egg yolk antibodies against a conserved mammalian protein. FASEB J. 4: 2528-2532.

Gignac SJ, Vo NTK, Mikhaeil MS, Alexander JAN, MacLatchy DL, Schulte PM, and Lee LEJ. 2014. Derivation of a continuous myogenic cell culture from an embryo of common killifish, *Fundulus heteroclitus*. COMP BIOCHEM PHYS A. 175: 15-27.

Gradin K, McGuire J, Wenger RH, Kvietikova I, Fhitelaw ML, Toftgard R, Tora L, Gassmann M, and Poellinger L. 1996. Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. MOL CELL. 16: 5221-5231.

Hatta H, Tsuda K, Akachi S, Kim M, and Yamamoto T. 1993. Productivity and some properties of egg yolk antibody (IgY) against human rotavirus compared with rabbit IgG. BIOSCI BIOTECH BIOCH. 57: 450-454.

Hill JD. 2013. Validation of antibodies used to study hypoxia inducible factors in two species of *Fundulus*. Thesis. University of New Orleans. Paper 1636.

Hochachka PW and Somero GN. 2002. Biochemical Adaptation: Mechanism and Process in Physiological Evolution. Oxford University Press, New York. 466 pp.

Huang LE, Gu J, Schau M, and Bunn HF. 1998. Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. PNAS. 95: 7987-7992.

Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, and Gassmann M. 2001. Induction of HIF-1α in response to hypoxia is instantaneous. FASEB J.15: 1312-1314.

Kaelin WG. 2005. Proline hydroxylation and gene expression. ANNU REV BIOCHEM. 74: 115–128.

Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, Tanaka H, and Poellinger L.1998. Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1α. EMBO J. 17: 6573-6586.

Law SHW, Wu RSS, Ng PKS, Yu RMK, and Kong RYC. 2006. Cloning and expression analysis of two distinct HIF-alpha isoforms – gcHIF-1alpha and gcHIF-4alpha – from the hypoxia-tolerant grass carp, *Ctenopharyngodon idellus.* BMC MOL BIOL. 7: 1-15.

Leslie GA and Clem LW. 1969. Phylogeny of immunoglobulin structure and function III. Immunoglobulins of the chicken. JEM. 130: 1337-1352.

Love JW and Rees BB. 2002. Seasonal differences in hypoxia tolerance in Gulf killifish, *Fundulus grandis* (Fundulidae). ENVIRON BIOL FISH. 63: 103-115.

Mahon P, Hirota K, and Semenza GL. 2001. FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity. GENE DEV. 15: 2675-2686.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, and Ratcliffe PJ. 1999.The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. NATURE. 399: 271-275.

McIntosh BE, Hogenesch JB, and Bradfield CA. 2010. Mammalian Per-Arnt-Sim proteins in environmental adaptation. ANNU REV PHYSIOL.72: 625-645.

Minet E, Mottet D, Michel G, Roland I, Raes M, Remacle J, and Michiels C. 1999. Hypoxia-induced activation of HIF-1: role of HIF-1α-Hsp90 interaction. FEBS LETT. 460: 251–256.

Nikinmaa M and Rees BB. 2005. Oxygen-dependent gene expression in fishes. AM J PHYSIOL-REG I. 288: R1079-R1090.

Pollock, MS, Clarke, LMJ, and Dube, MG. 2007. The effects of hypoxia on fishes: from ecological relevance to physiological effects. ENVIRON REV. 15: 1-14.

Rabalais NN, Diaz RJ, Levin LA, Turner RE, Gilbert D, and Zhang J. 2010. Dynamics and distribution of natural and human-caused hypoxia. BIOGEOSCIENCES. 7: 585-619.

Rahman MS and Thomas P. 2007. Molecular cloning, characterization and expression of two hypoxia-inducible factor alpha subunits, HIF-1α and HIF-2α, in a hypoxia-tolerant marine teleost, Atlantic croaker (*Micropogonias undulatus*). GENE. 396: 273–282.

Richards JG, Farrell AP, Brauner CJ. 2009. Fish Physiology: Hypoxia. Vol 27. Academic Press.

Rissanen E, Tranberg HK, Sollid J, Nilsson GE, and Nikinmaa M. 2006. Temperature regulates hypoxia-inducible factor-1 (HIF-1) in a poikilothermic vertebrate, crucian carp (*Carassius carassius*). J EXP BIOL. 209: 994-1003.

Rytkönen KT, Williams TA, Renshaw GM, Primmer, GR, Nikinmaa M. 2011. Molecular evolution of the metazoan PHD- HIF oxygen- sensing system. MOL BIOL EVOL. 28: 1913-1926.

Sambrook J, Fritsch EF, and Maniatis T. 1989. Molecular Cloning: A Laboratory Manual, 2nd Edition. CSHL Press. New York.

Semenza GL.1998. Hypoxia-inducible factor 1: master regulator of O_2 homeostasis. CURR OPIN GENET DEV. 8: 588-594.

Semenza GL.1999. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. ANNU REV CELL DEV BIOL. 15: 551-578.

Semenza GL. 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J APPL PHYSIOL. 88: 1474-1480.

Semenza GL. 2009. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. PHYSIOLOGY. 24: 97-106.

Semenza GL. 2010. Oxygen homeostasis. WILEY INTERDISCIP REV SYST BIOL MED. 2: 336-361.

Semenza GL. 2014. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. ANNU REV PATHOL-MECH. 9: 47-71.

Semenza GL and Wang GL. 1992. A nuclear factor induced by hypoxia via *de novo* protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. MOL CELL. 12: 5447–5454.

Shen RJ, Jiang XY, Pu JW, and Zou SM. 2010. HIF-1α and 2α genes in a hypoxiasensitive teleost species *Megalobrama amblycephala*: cDNA cloning, expression and different responses to hypoxia. COMP BIOCHEM PHYS B. 157: 273-280.

Smith PK, Krohn RI, Hermanson, GT, Mallia, AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, and Klenk DC.1985. Measurement of protein using bicinchoninic acid. ANAL BIOCHEM. 150: 76–85.

Soitamo AJ, Rabergh CMI, Gassmann M, Sistonen L, and Nikinmaa M. 2001. Characterization of a hypoxia-inducible Factor (HIF-1α) from rainbow trout, accumulation of protein occurs at normal venous oxygen tension. J BIOL CHEM. 276: 19699-19705.

Sollid J, Rissanen E, Tranberg HK,Thorstensen T, Vuori KAM, Nikinmaa M, and Nilsson GE. 2006. HIF-1α and iNOS levels in crucian carp gills during hypoxia-induced transformation. J COMP PHYSIOL B.176: 359-369.

Song CS, Yu JH, Bai DH, Hesler PY, and Kim KH. 1985. Antibodies to the alpha-subunit of insulin receptor from eggs of immunized hens. J IMMUNOL. 135: 3354-3359.

Stierhoff KL, Targett TE, and Grecay PA. 2003. Hypoxia tolerance of the mummichog: The role of access to the water surface. J FISH BIOL. 63: 580-592.

Terova G, Rimoldi S, Cora S, Bernardini G, Gornati R, and Saroglia M. 2008. Acute and chronic hypoxia affects HIF-1α mRNA levels in sea bass (*Dicentrarchus labrax*). AQUACULTURE. 279: 150-159.

Thomas P, and Rahman MS. 2009. Biomarkers of hypoxia exposure and reproductive function in Atlantic croaker: A review with some preliminary findings from the northern Gulf of Mexico hypoxic zone. J EXP MAR BIOL ECOL. 381: S38-S50.

Wannamaker CM and Rice JA. 2000. Effects of hypoxia on movements and behavior of selected estuarine organisms from the southeastern United States. J EXP MAR BIOL ECOL. 249: 145-163.

Wenger RH and Gassmann M.1997. Oxygen(es) and the hypoxia-inducible factor-1. BIOL CHEM. 378: 609-616.

Wetzel, RG. 2001. Limnology: Lake and River Ecosystems (3rd ed.). San Diego, CA: Academic

Press.

Zhang P, Lu L, Yao Q, Li Y, Zhou J, Liu Y, and Duan C. 2012. Molecular, functional, and gene expression analysis of zebrafish hypoxia-inducible factor-3α. AM J PHYSIOL-REG I. 303: R1165-R1174.

Table 1: Results from database searching of mass spectrometry analyses of ion-exchange elution fractions (Figure 4C). Matches with p-value less than 10^{-8} are shown.

Gel Band	Apparent MW (kD)	Top hits	p-value	Actual MW (kD)
1	16	*Fundulus heteroclitus* HIF-1α	2.20 E-15	83.9
2	21	*cyclic AMP receptor protein	1.40 E-13	23.6
2	21	*orf, conserved hypothetical protein	3.10 E-10	21.2
3	54	*pyruvate kinase	3.50 E-13	51.3
3	54	*glucose-6-phosphate 1-dehydrogenase	1.30 E-08	55.6
4	70	*hypothetical protein b2255	4.90 E-15	74.2
4	70	*molecular chaparone DnaK	1.30 E-10	69
5	25	*hypothetical protein b2255	1.27 E-12	74.2
5	25	*FKBP-type peptidyl-prolyl cis-trans isomerase	9.30 E-12	20.8
6	65	*D-fructose-6-phosphate amidotransferase	1.33 E-14	66.8
6	65	*hypothetical protein b2255	1.07 E-11	74.2

*from *E.coli* database

Table 2: Purification of polyhistidine-tagged HIF-1α peptide from 4 x 1- l *E.coli* cultures.

Sample	**Protein concentration (mg/ml)**	**Total volume (ml)**	**Total protein (mg)**
Lysate (supernatant)	5.08	200	1016
Ni-NTA eluate	0.81	15	12.15
Ion exchange eluate	32.61	0.205*	6.68
Gel filtration eluate	4.74	0.54*	2.56

*volumes after sample concentration and dialysis.

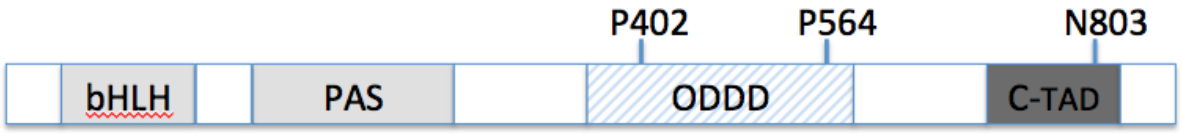

Figure 1: Schematic representation of HIF-1α domains. Basic helix-loop-helix (bHLH), Per-ARNT-Sim (PAS), C-terminal transactivation (C-TAD), oxygen-dependent degradation domain (ODDD), Proline (P) and Asparagine (N). Amino acid numbers from human HIF-1α. Figure modified from Kaelin, 2005.

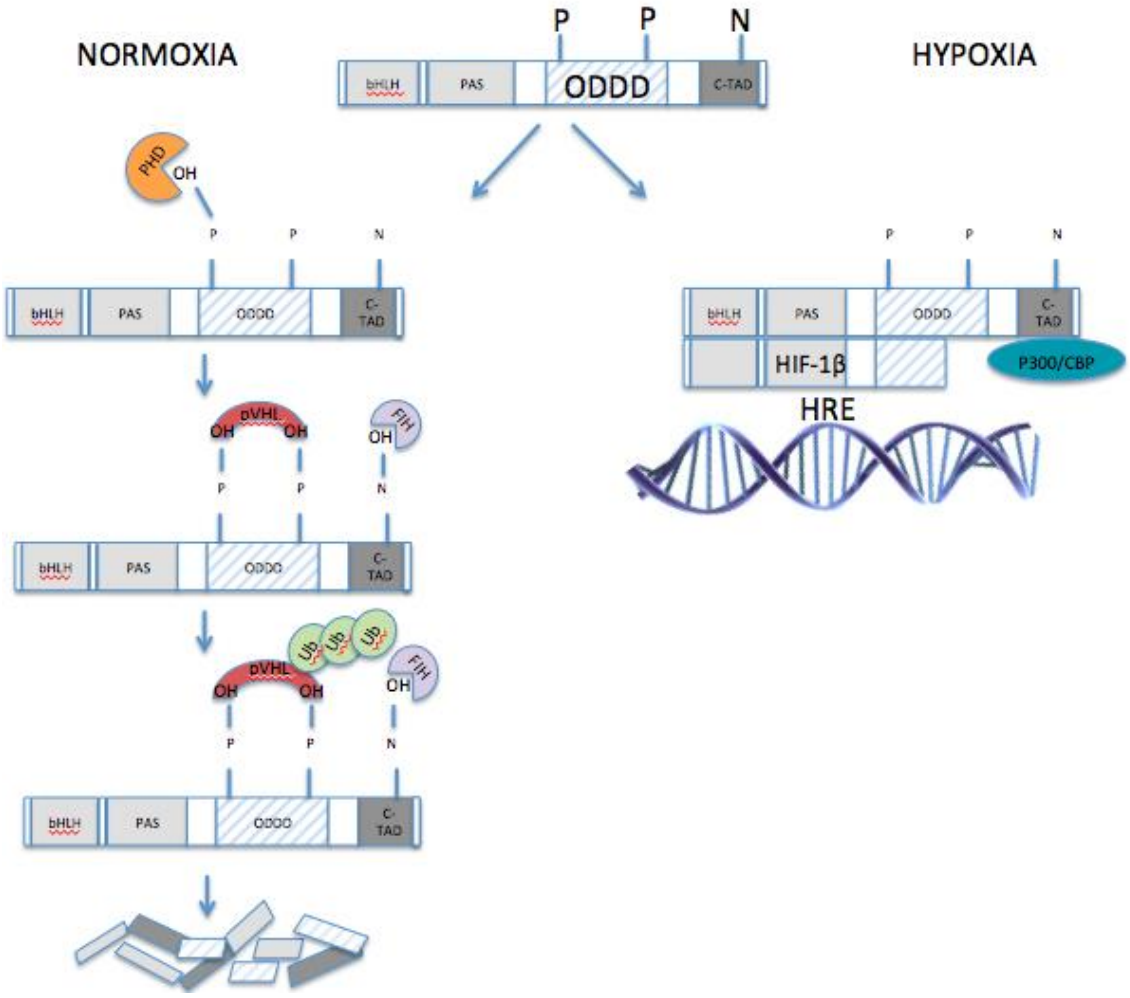

Figure 2: Schematic representation of hypoxia-inducible factor (HIF) regulation under normoxic and hypoxic conditions. Under normoxia, prolyl hydroxylase (PHD) enzymes hydroxylate proline (P) residues 402 and 564, targeting HIF-1α for ubiquitination (Ub) by pVHL, and degradation by the 26S proteasome. In addition, factor-inhibiting HIF (FIH) hydroxylates asparagine (N) residue 803 preventing recruitment of coactivators. Under hypoxia, PHD and FIH activities decrease and HIF-1α accumulates, dimerizes with HIF-1β, and recruits coactivators p300 and CBP. The complex translocates to the nucleus and binds to hypoxia response elements (HRE) on specific target genes, resulting in increased rates of transcription. Amino acid numbers from human HIF-1α.

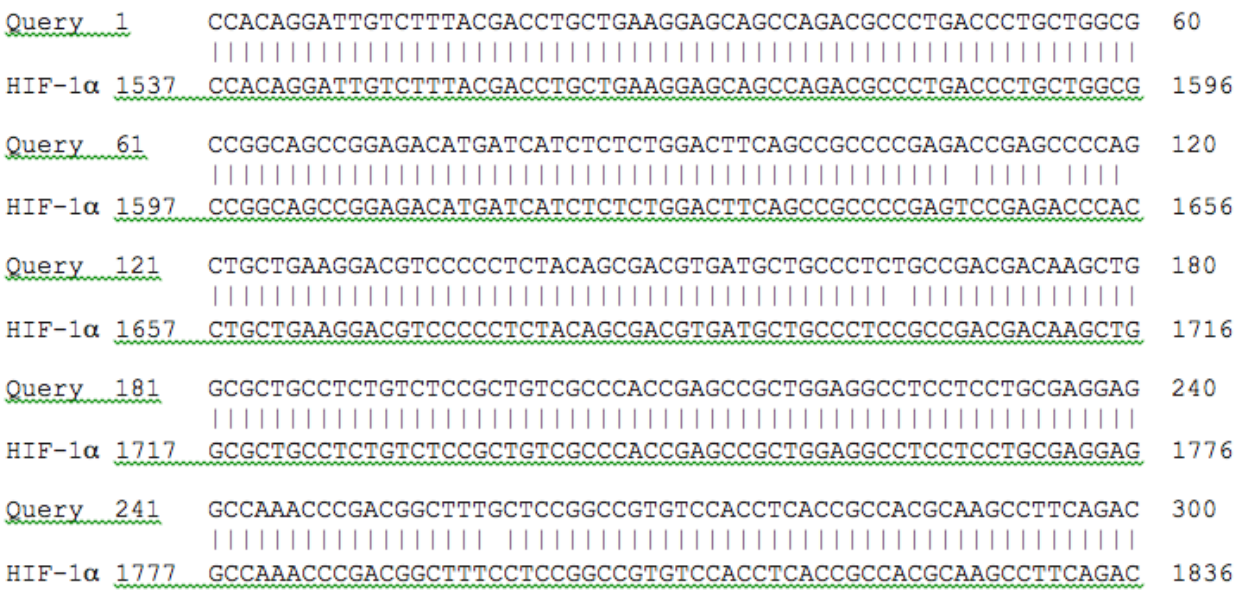

Figure 3: Sequence alignment of cloned cDNA with *F. heteroclitus* HIF-1α.

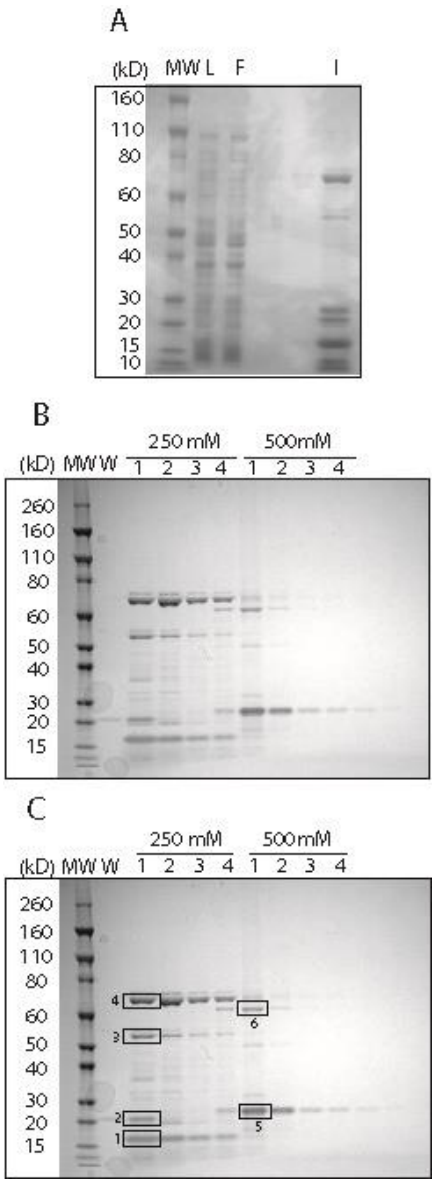

Figure 4: (A) SDS-PAGE gel of Ni-NTA fractions stained for total protein. MW is molecular weight marker; L is total cell lysate added to the column; F is flow-through; I is 200 mM imidazole fraction. (B) SDS-PAGE gel of ion-exchange fractions stained for total protein. The column was eluted with Buffer I containing 250 mM and 500 mM NaCl (showing fractions 1- 4 for each). (C) Same gel as in part (B) indicating bands (1-6) excised for mass spectrometry analysis.

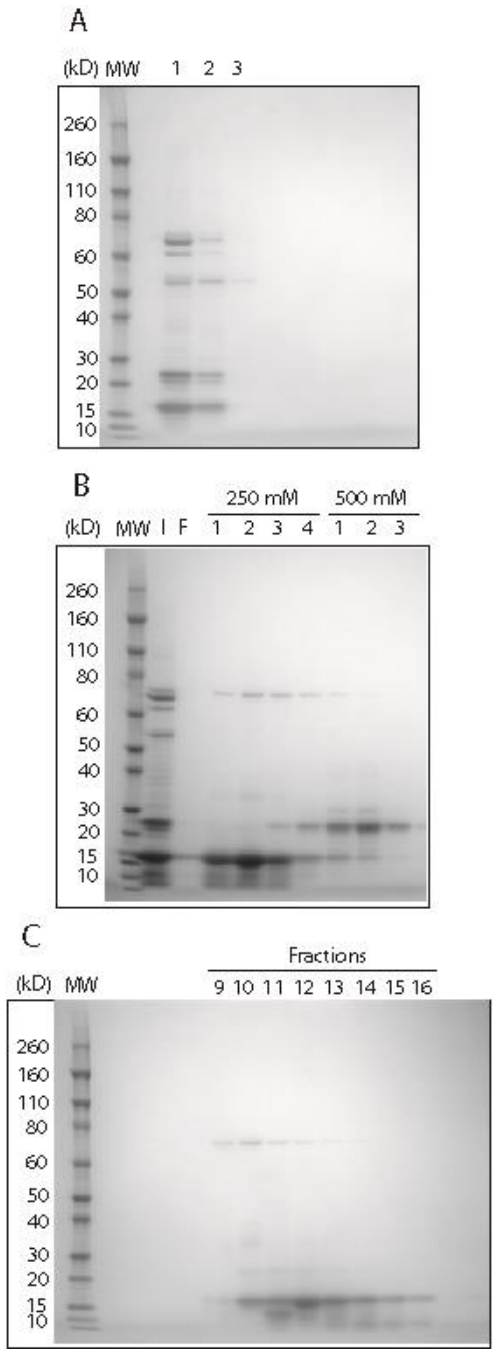

Figure 5: (A) SDS-PAGE gel showing the polyhistidine-tagged HIF-1α peptide by Ni-NTA chromatography. Imidazole elution fractions 1- 3 show a prominent band at 16 kD. (B) SDS-PAGE gel of ion-exchange chromatography results. I is the eluate from the Ni-NTA column that was applied to the ion-exchange column; F is flow-through. The column was eluted with Buffer I containing 250 mM and 500 mM NaCl (showing fractions for each). (C) SDS-PAGE gel of gel filtration of the pooled 250 mM NaCl fractions from ion-exchange chromatography. The 16 kD band corresponding to polyhistidine-tagged HIF-1α peptide eluted in fractions 10-16.

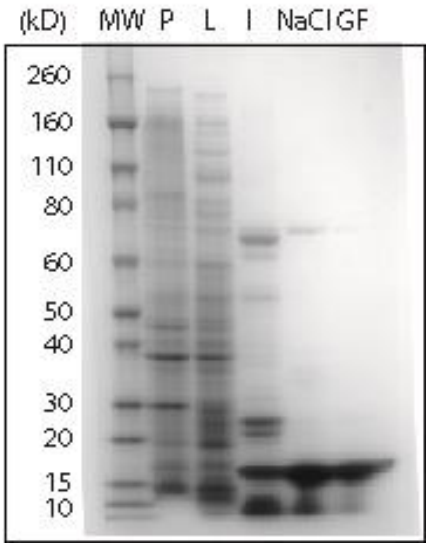

Figure 6: SDS-PAGE gel showing the overall purification of polyhistidine-tagged HIF-1α peptide. (P) Cell pellet; (L) Cell lysate supernatant added to Ni-NTA column; (I) eluate from Ni-NTA column; (NaCl) eluate from ion-exchange column (250 mM fractions pooled); (GF) eluate from gel filtration column. In GF, the only band present corresponds to the polyhistidine-tagged HIF-1α peptide.

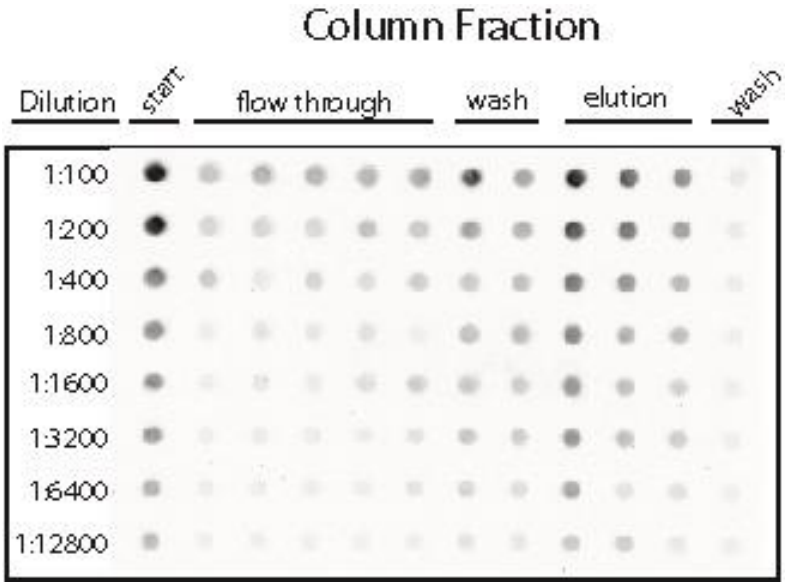

Figure 7: Dot blot analysis of HIF-1α antibody purification. Start is the total IgY applied to the affinity column. Flow-through is what did not bind to the column. Wash with TBS. Elution of column with 0.1 M glycine pH 2.5. Followed with wash with TBS. Dilution corresponds to serial dilution of all fractions. The starting volume was 10-12 ml of IgY (varied among experiments), and all column fractions were 2 ml each.

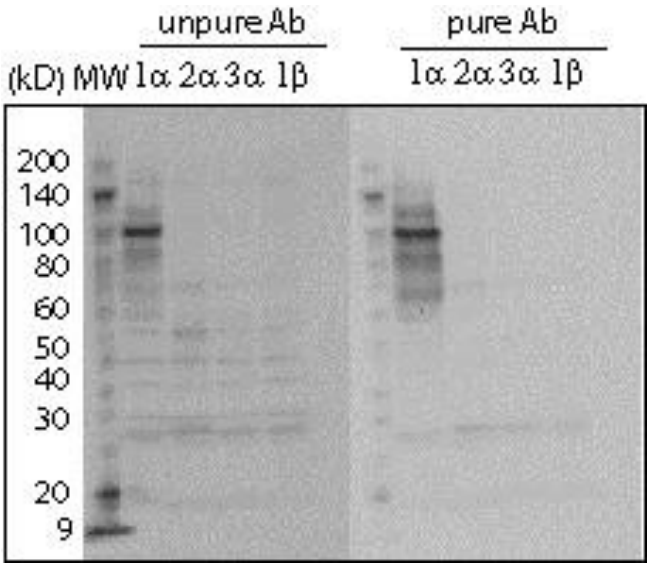

Figure 8: Western blot analysis of *in vitro*-expressed full length of HIF-1α (1α), HIF-2α (2α), HIF-3α (3α) and ARNT (β), all at a volume of 3 μl of IVTT full length protein, probed with unpurified HIF-1α antibody (1:1000) or purified HIF-1α antibody (1:500).

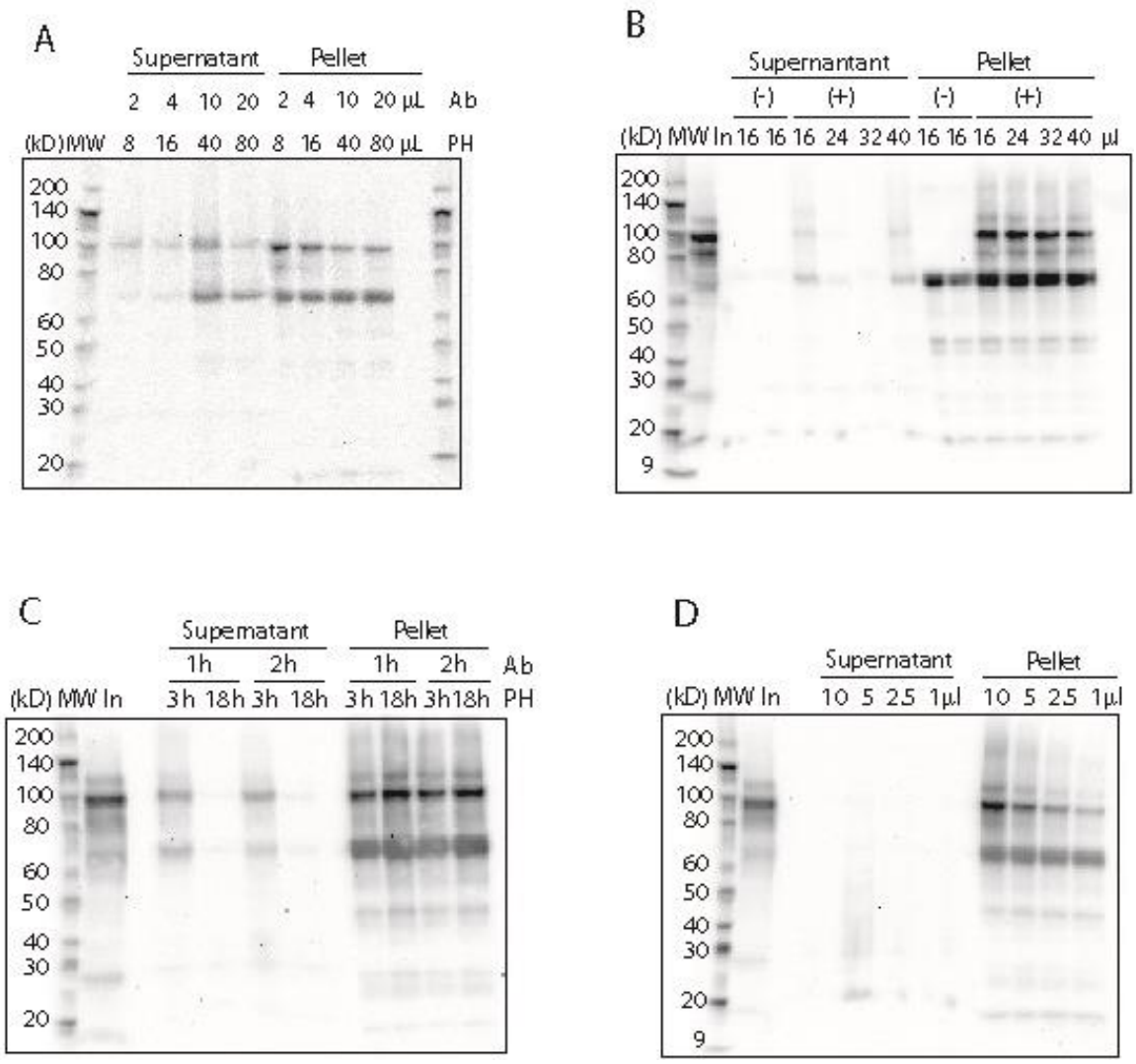

Figure 9: Optimization of immunoprecipitation. In is for the positive control lane contained 4 µl IVTT full length HIF-1α. (A) Antibody variation at a constant Ab: PrecipHen ratio. All reactions contained 10 µl IVTT HIF-1α lysate. (B) PrecipHen variation. Control reactions (-), the first had no added lysate and the second is an unprogrammed rabbit reticulocyte lysate. Positive reactions (+) contain 16, 24, 32, and 40 µl of PrecipHen. All reactions had 4 µl Anti-HIF-1α antibodies and 10 µl IVTT HIF-1α lysate. (C) Incubation time variation. Primary antibody incubation: 1 and 2 h. PrecipHen incubation: 3 and 18 h. All reactions had 4 µl Anti-HIF-1α antibodies, 20 µl of PrecipHen, and 10 µL IVTT HIF-1α lysate. (D) Variation in added IVTT HIF-1α lysate: 1, 2.5, 5, and 10 µl IVTT HIF-1α lysate.

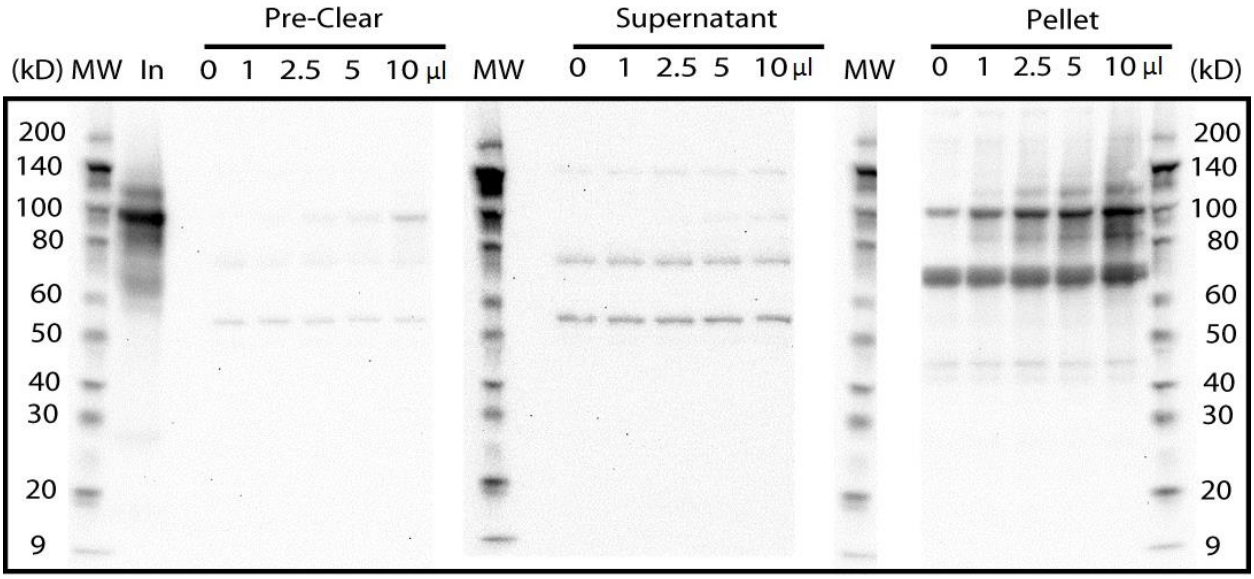

Figure 10: Immunoprecipitation of extracts from normoxic KFE-5 cells using 0.8 mg/ml of protein per reaction and IVTT HIF-1α lysate added at: 1, 2.5, 5, and 10 µl. "In" is the positive control lane containing 4 µl IVTT HIF-1α lysate. The pre-clear step was included to remove non-specific proteins. An endogenous HIF-1α band was detected in the KFE-5 extract without added IVTT HIF-1α lysate (0 µl, Pellet).

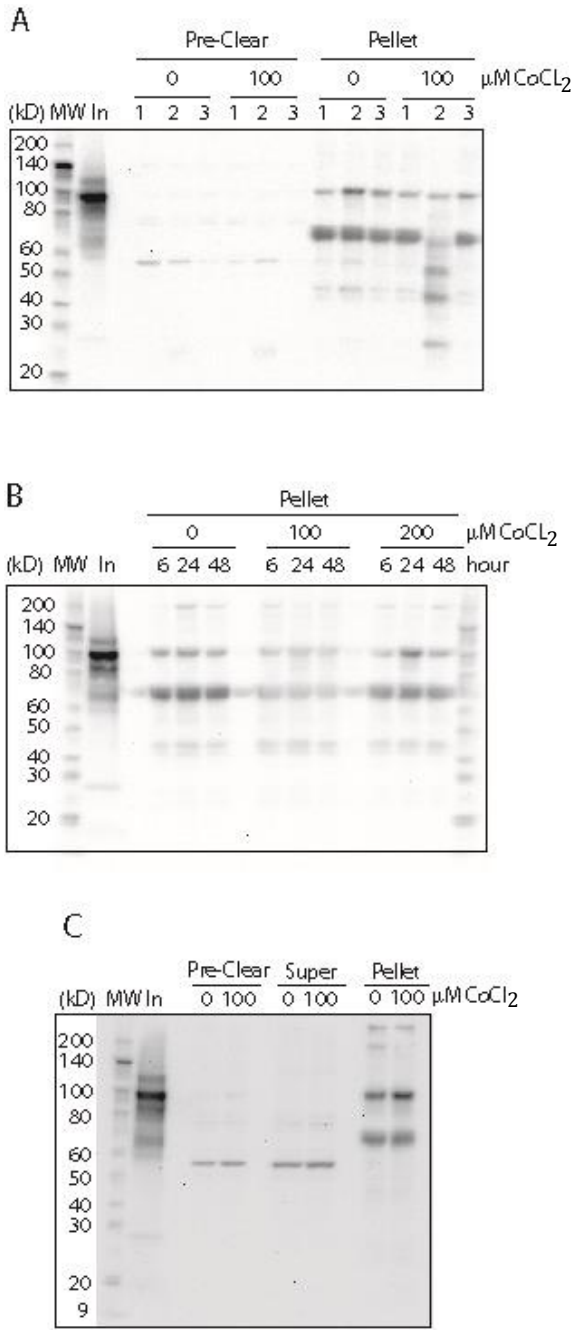

Figure 11: Immunoprecipitation of KFE-5 cell extracts after $CoCl_2$ stimulation. (A) Triplicate cell culture flasks (1, 2, 3) were incubated with or without 100 µM $CoCl_2$ for 24 h. IP reactions contained 0.8 mg/ml protein. (B) KFE-5 cells were incubated at 0, 100, or200 µM $CoCl_2$ for 6, 24, or 48 h. IP reactions had 0.8 mg/ml protein except the 6 h 100 µM sample was 0.6 mg/ml due to limited sample. (C) KFE-5 cell lysates from 0 µM $CoCl_2$ (6 and 24 h combined) and 100 µM $CoCl_2$ (24 h) were immunoprecipitated at 2.15 mg/ml protein.

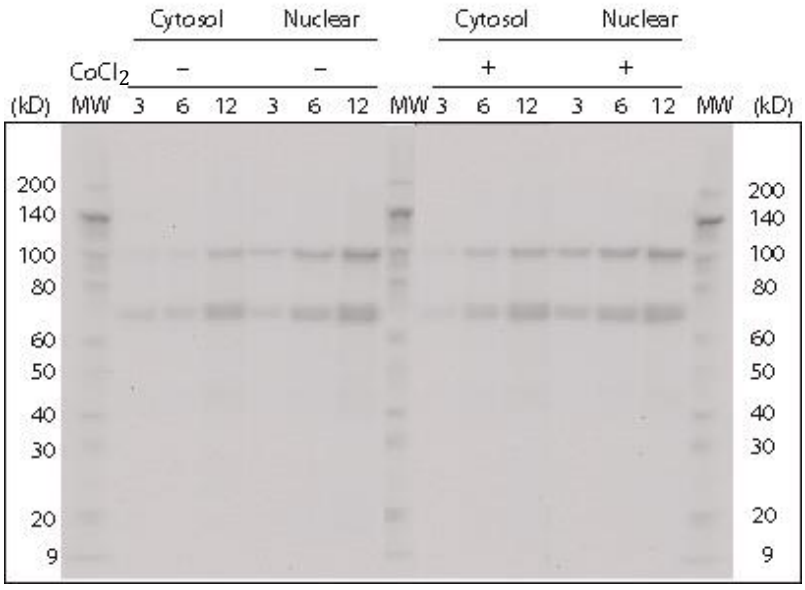

Figure 12: Immunoprecipitation of KFE-5 cell extracts with or without 24 h 100 μM $CoCl_2$ treatment. Prior to IP, lysates were separated into cytosol and nuclear fractions. The volume of each IP reaction loaded on the gel was 3μl (3), 6μl (6) or 12 μl (12) for each sample.

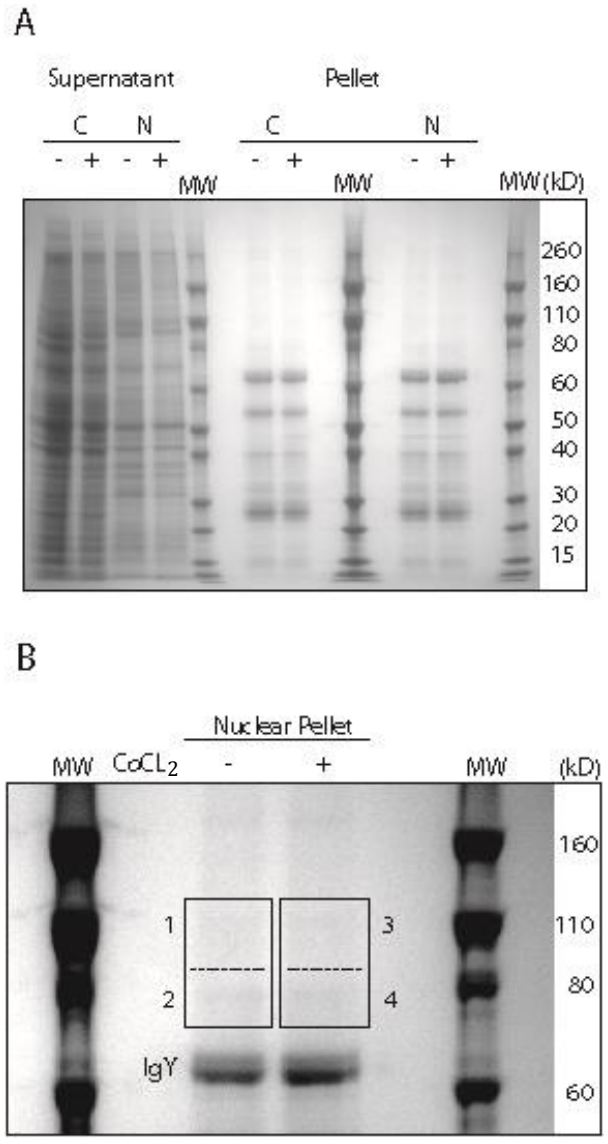

Figure 13: Total protein stain of KFE-5 cell extracts with or without 24 h 100 μM $CoCl_2$ treatment. (A) Colloidal Coomassie stained SDS-PAGE gel of cytosolic (C) and nuclear (N) fractions from 0 μM $CoCl_2$ (-) and 100 μM $CoCl_2$ (+) treated cells. (B) Magnification of the gel region containing the bands cut for mass spectrometry analysis (boxes labeled 1-4). IgY is chicken immunoglobulins.

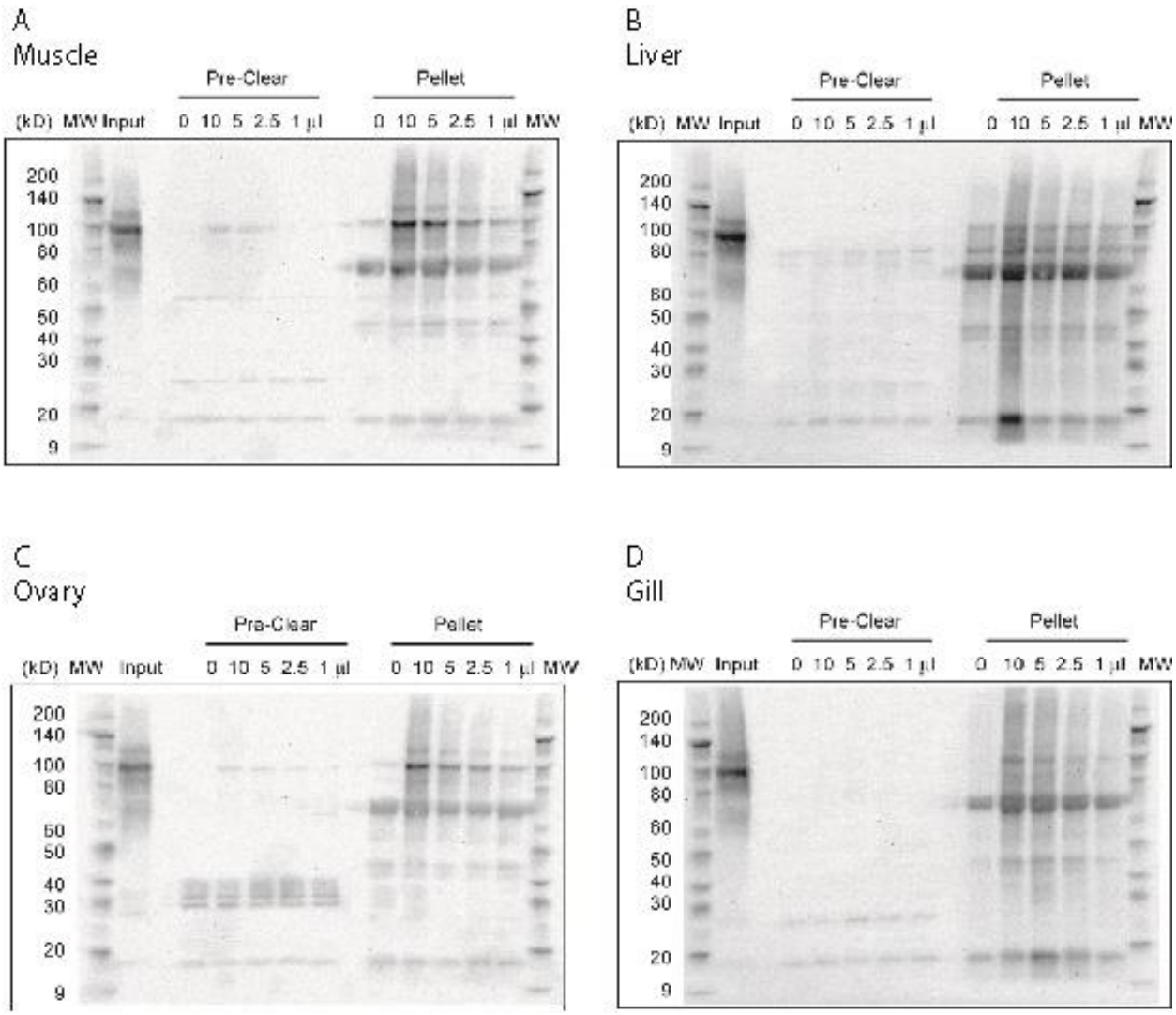

Figure 14: Immunoprecipitation of tissue lysates from normoxic *F. grandis*. Reactions contained 2 mg/ml of tissue lysate protein and IVTT HIF-1α as indicated. (A) Muscle (B) Liver (C) Ovary (D) Gill. HIF-1α migrates at 100 kD (see input) and a band of this size was observed in tissue extracts without added IVTT HIF-1α (0 µl).

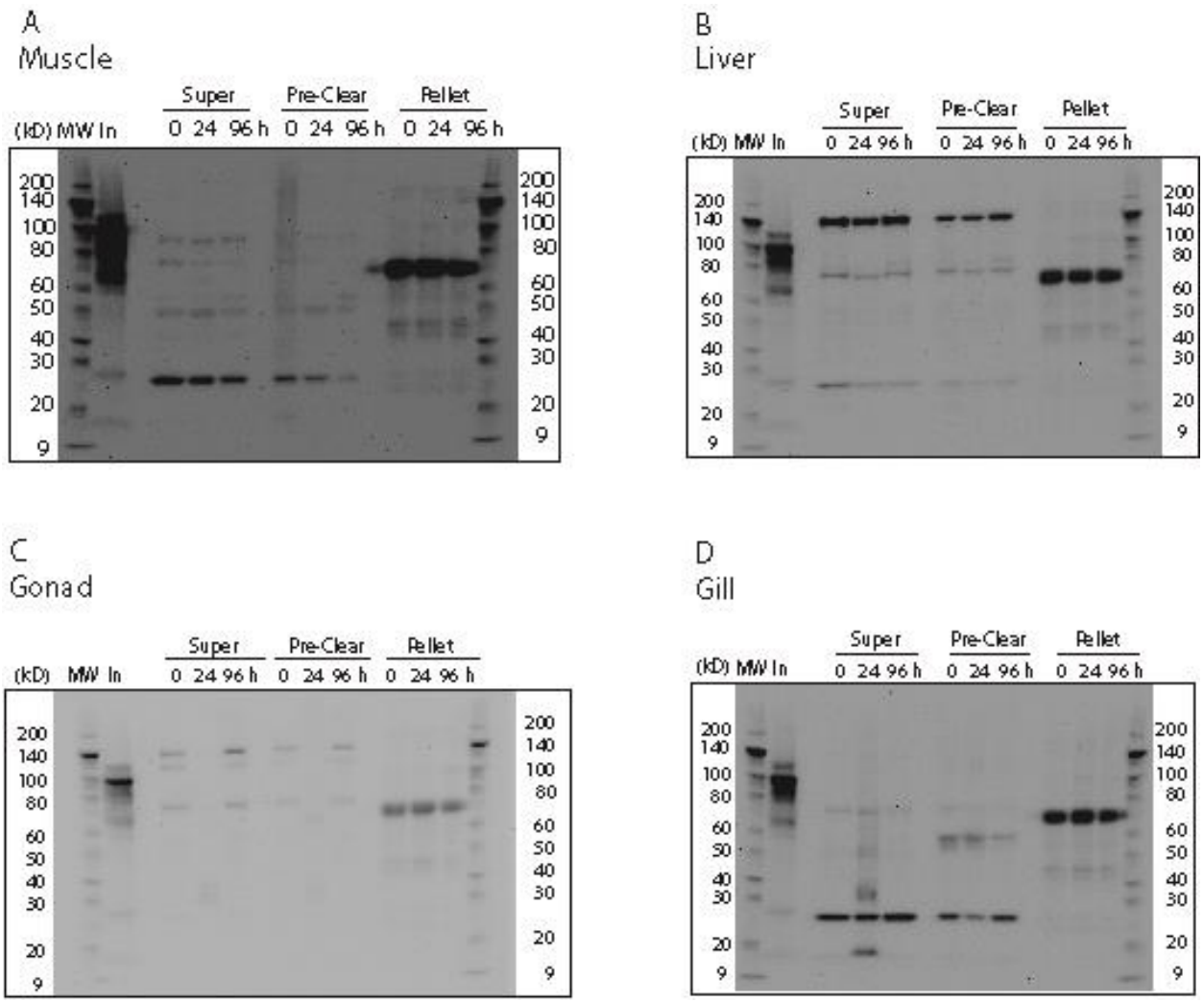

Figure 15: Immunoprecipitation of tissue lysates from *F. grandis* held for 0, 24, or 96 h at ~ 0.9 mg/l DO (11% of the air-saturated value). Reactions contained 2 mg/ml of lysate protein. (A) Muscle (B) Liver (C) Gonad (D) Gill. HIF-1α migrates at 100 kD (see input).

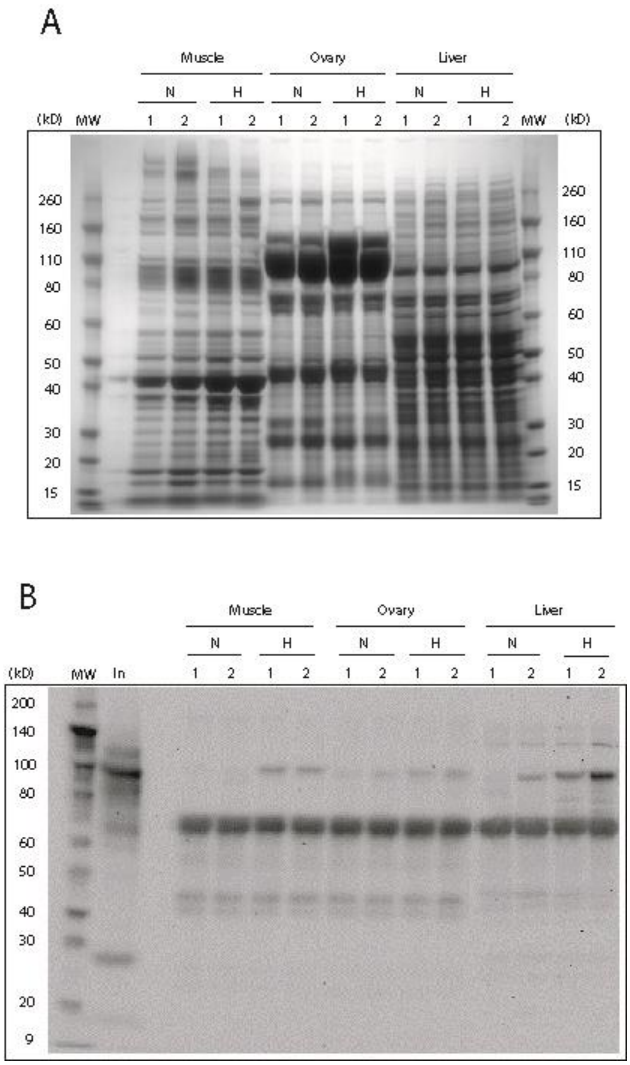

Figure 16: Results of IP of tissues from *F. grandis* held under normoxia (N) or hypoxia (H). Reactions contained 2 mg/ml of lysate protein. Fish (*N*=2 for each treatment) incubated for 24 h at ~ 7.9 mg/l (99% air saturation) for normoxia or 0.6 mg/l (7.5% air saturation) for hypoxia. (A) SDS-PAGE gel stained for total protein with Colloidal Coomassie. (B) Immunoprecipitated proteins detected by western blotting. Note the band at 100 kD that is more abundant in hypoxic samples.

6. VITA

Janet Gonzalez-Rosario was born in Camaguey, Cuba on December 17, 1977 as the daughter of Jose D. Gonzalez and Olga Gonzalez. On May 5, 1980 during the Mariel Boatlift she arrived on the shores of Miami, Florida: she was two years old. She attended American Senior High in Hialeah, Florida and graduated in May 1995. She is the wife of Michael S. Rosario and mother to Samantha, Vanessa, and Michael L. Rosario. After moving around the country for her husband's career and attending several colleges and universities, in August 2011, she enrolled at University of New Orleans. In May 2012, after being awarded the Undergraduate, Research and Mentoring Award funded by the National Science Foundation, she joined the research laboratory of Dr. Bernard Rees. In May 2013, she received a Bachelor of Science degree in Biology. She was admitted into Graduate School in the Department of Biological Sciences at the University of New Orleans in August 2013, continuing her research in the laboratory of Dr. Bernard Rees. She received a Master of Science degree in May 2016.