THE MAMMALIAN HYPOXIA RESPONSE PATHWAY: REGULATION OF HIF AND HIF PROLYL HYDROXYLASES

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DEDICATION

To my parents Ayper Ozer and Salihdin Ozer,

and to my wife Mine Ozer.

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by

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DISSERTATION

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Cells exposed to hypoxia –limited oxygen availability– initiate an adaptive response orchestrated by a transcription factor called Hypoxia Inducible Factor (HIF). HIF is composed of an oxygen-sensitive α-, and an oxygen-insensitive β-subunit (ARNT). The stability and transcriptional activity of HIF-α are controlled by two different Fe(II)- and 2-oxoglutarate-dependent dioxygenases that utilize molecular oxygen during hydroxylation of HIF α-subunit. When oxygen levels are sufficient (normoxia), HIF Prolyl Hydroxylases (HPH-1, -2, and -3) hydroxylate the Oxygen-dependent Degradation Domain (ODD) of HIF-α targeting it to ubiquitin-mediated proteosomal degradation. Factor Inhibiting HIF 1 (FIH-1,

an asparaginyl hydroxylase), on the other hand, hydroxylates C-terminal Transactivation Domain (CTAD) thereby abolishing recruitment of transcriptional co-activators by HIF-α. However, under hypoxic conditions, both hydroxylations are diminished allowing HIF-α to escape degradation and induce transcription by associating with co-activators. Because of its critical role as an oxygen sensor, we studied HIF Prolyl Hydroxylase 2 (HPH-2) and focused on protein-protein interactions expecting that some of the interacting proteins might regulate its function. We characterized the function of a HPH-2 interacting protein identified in yeast two-hybrid screen; Inhibitor of Growth 4 (ING4) –a candidate tumor suppressor protein–, and showed that ING4 represses HIF transcriptional activity under hypoxia in a chromatin-dependent manner. Recruitment of ING4 to alter HIF transcriptional activity represents a novel function of HPH-2. To shed some light on the mechanism of this transcriptional repression, we purified ING4 containing co-repressor complex containing MYST2 and JADE3. Furthermore, we showed that ING4 and MYST2 targets not only HIF but also NF-κB transcription factor, a previously identified target of ING4, perhaps misregulation of which in the absence of functional ING4 protein contributes to tumor progression. Moreover, we identified additional HPH-2 interacting proteins and found that HPH enzymes can be modified by Protein Arginine Methyltransferase 1 (PRMT1) *in vitro*. Inhibition of methyltransferases *in vivo* further stabilized and activated HIF-1α suggesting a role for methyltransferases in regulation of HIF that might be mediated through HPH enzymes. Methylation of HPH enzymes, the first identified post-translational modification of these enzymes, adds another layer of complexity to the regulation of HIF and it may serve as an interface between the hypoxia response pathway and other signaling pathways.

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Be The Best of Whatever You Are
(by Douglas Malloch)

If you can't be a pine on the top of the hill,
Be a scrub in the valley - but be
The best little scrub by the side of the rill;
Be a bush if you can't be a tree.
.
.
It isn't by size that you win or you fail -
Be the best of whatever you are!

I tried to become the best of who and what I am. But none of this would ever be possible without the help of the following people, which I would like to thank.

It is difficult to overstate my gratitude to the mastermind and the driving force behind all of this work, my Ph.D. supervisor Dr. Richard K. Bruick. Since the first day I joined his lab, I have always found him encouraging, enthusiastic, inspirational, and demanding, which I have benefited tremendously. He has been a great mentor for me; he literally sat with me and taught me how to design and execute experiments, how to interpret and follow up on my results, and numerous experimental shortcuts also known as “save a day plans”. I am thankful for his insightful suggestions, sound advice, and perfectionistic comments on not only my scientific skills but also on my presentation and writing skills. I am also very thankful for the freedom, which I enjoyed especially over the last two years, to explore new ideas and to pursue alternative projects. This was purposely done by Rick to make me independent scientist, and this by itself shows how good a mentor and how caring he has been for me. Also, I am grateful for his help to get me registered to a meeting held in Germany, and allowing me stay there at the time of my mother’s sickness. I also thank Rick

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"...we have no knowledge except what You have taught us.

Surely You are the All-Knowing, the All-Wise." (Al-Baqarah, 32)

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Ozer A. and Bruick R.K. Non-heme dioxygenases: cellular sensors and regulators jelly rolled into one? *Nat. Chem. Biol.* 3 (3):144-153, 2007.

Ozer A. and Bruick R.K. Regulation of HIF by prolyl hydroxylases: recruitment of the candidate tumor suppressor protein ING4. *Cell Cycle* 4 (9):1153-1156, 2005.

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LIST OF FIGURES

LIST OF TABLES

LIST OF DEFINITIONS

AD	Activation Domain
AdOx	Adenosine-2',3'-dialdehyde
AhR	Arylhydrocarbon Receptor
AK3	Adenylate Kinase 3
ALDA	Aldolase A
ARNT	Arylhydrocarbon Receptor Nuclear Translocator
bHLH	Basic-helix-loop-helix
BRG1	BRAHMA Related Gene 1
BRMS1	Breast cancer Metastasis Suppressor 1
BSA	Bovine Serum Albumin
CBP	CREB Binding Protein
CBP	Calmodulin Binding Peptide
Ch-IP	Chromatin-Immunoprecipitation
co-IP	Co-Immunoprecipitation
COX2	Cytochrome c Oxidase 2
CTAD	C-terminal Transactivation Domain
DBD	DNA Binding Domain
DFO	Desferrioxamine
DMOG	Dimethyloxallyl Glycine
EGLN#	EGL Nine homolog #
ELISA	Enzyme-Linked ImmunoSorbent Assay

EPAS-1	Endothelial PAS domain protein 1
EPO	Erythropoietin
FIH-1	Factor Inhibiting HIF 1
FKBP8	FK-506 Binding Protein 8
GLUT#	Glucose Transporter #
GPS1	G-protein Pathway Supressor 1
GST	Glutathione-S-Transferase
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HIF	Hypoxia Inducible Factor
hnRNP	Heterogeneous Nuclear Ribonucleoprotein
HPH-#	HIF Prolyl Hydroxylase #
HPLC	High Preformance Liquid Chromatography
HRE	Hypoxia Response Element
HSP90	Heat Shock Protein 90
IκB	Inhibitor κB
IKK	IκB Kinase
IL#	Interleukin #
ING#	Inhibitor of Growth #
JADE#	Gene for Apoptosis and Differentiation in Epithelia #
LDHA	Lactate Dehydrogense A
LZL	Leucine Zipper-like

MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time Of Flight
MBP	Maltose Binding Protein
MEP50	Methylosome Protein 50
MS	Mass Spectrometry
MyBP	Myelin Basic Protein
MYND	MTG8, Nervy, DEAF-1
MYST2	MOZ,YBF2/SAS3, SAS2 and TIP60 histone acetyltransferase 2
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor κB
NLS	Nuclear Localization Signals
NTAD	N-terminal Transactivation Domain
NTAP	N-terminal TAP-tag
ODD	Oxygen-dependent Degradation Domain
OS-9	Osteosarcoma overexpressed 9
PAGE	Polyacrylamide Gel Electrophoresis
PAS	PER, ARNT, and SIM
PCNA	Proliferating Cell Nuclear Antigen
PCR	Potential Chromatin Regulatory
PGK1	Phosphoglycerate Kinase 1
PHD	Plant Homeodomain
PHD#	Prolyl Hydroxylase Domain #
PIP	PCNA Interacting Protein

PRMT#	Protein Arginine Methyltransferase #
PPIase	Peptidyl-prolyl Isomaerase
PtdInsP	Phosphotidylinositolphosphate
pVHL	Product of von Hippel-Lindau
qRT-PCR	Quantitative Real-time PCR
ROS	Reactive Oxygen Species
RPA#	Replication Protein A #
SAM	S-Adenosyl-L-Methionine
SCCA1	Squamous Cell Carcinoma Antigen 1
SDS	Sodium Dodecyl Sulfate
SIAH1	Seven In Absentia Homolog 1
SIAH2	Seven In Absentia Homolog 2
siRNA	Short Interfering RNA
SWI/SNF	Switching/Sucrose Non-fermenting
TAP	Tandem Affinity Purification
TCA	Tricarboxylic Acid
TRiC	TCP-1 Ring Complex
VEGF	Vascular Endothelial Growth Factor

CHAPTER ONE
Introduction

Life can be defined in many ways by many people from different walks of life. From the biochemist's point of view, life is practically defined as "a set of chemical reactions." While this five-word definition generates a connotation of simplicity, one must realize that a single subset of chemical reactions occurring within a cell possesses many complex interconnections between each step, as well as interconnections between entirely different sets of reactions. For example, proteins, encoded by the genetic material, function as the catalysts in many of these reactions such as the oxidation of carbon atom, and the synthesis of these proteins requires the polymerization of amino acids, it in itself a chemical reaction. While these sets of chemical reactions can possess seemingly endless layers of complexity, a unifying theme of these reactions is the requirement of free energy in one form or another to promote the formation of reaction products. In higher eukaryotes, the greatest source of energy production depends on oxygen as the terminal electron acceptor in oxidative phosphorylation by which chemical energy is finally converted into a biologically available form used to fuel lively reactions. Therefore, the statement "No oxygen, no life." is not too far from the truth, for higher eukaryotes, but not necessarily the case for prokaryotes.

As it is true for almost everything, there can be too much of a good thing. In the case of oxygen, high concentrations (hyperoxia) can be deleterious by causing oxidation of critical biomolecules including DNA and proteins, whereas limiting levels (hypoxia) leads to cessation of many processes, the most important being energy production. Therefore, all

living organisms must keep oxygen levels within a narrow range. Under physiological conditions, mammalian cells are exposed to oxygen levels that range anywhere between 13% at lung alveoli (Allen, Jones et al. 1984) to ~2% at medulla of kidney (Neuhofer and Beck 2005). These various oxygen levels are considered normal (normoxia) by cells of a given tissue. However, any reduction in these oxygen levels due to lower oxygen tension at high altitude (Lahiri, Roy et al. 2006) or due to decrease in oxygen supply as a result of pathophysiological conditions (Semenza 2000) such as pulmonary diseases, ischemia, and cancer is regarded as hypoxia. Similarly, cells grown in cell culture are adjusted to 21% oxygen while exposure to <5% oxygen is regarded as hypoxia (Jiang, Semenza et al. 1996).

Hypoxia response pathway and Hypoxia Inducible Factor (HIF)

Exposure to hypoxic conditions activates physiological adaptive responses including increase in the rate of lung ventilation and blood circulation to raise oxygen supply to meet cellular demand (Lahiri, Roy et al. 2006). At the same time, cellular processes are altered to adapt and survive with limited oxygen availability. Production of oxygen carrying cells, erythrocytes, is enhanced by secreted Erythropoietin (EPO), new blood vessel formation, angiogenesis, is induced by Vascular Endothelial Growth Factor (VEGF), and glycolysis is accelerated by upregulation of multiple proteins involved in this pathway (Glucose Transporter 1 (GLUT1) and 3 (GLUT3), Hexokinase 1 (HK1) and 2 (HK2), Aldolase A (ALDA), and Phosphoglycerate Kinase 1 (PGK1)) (Iyer, Kotch et al. 1998; Semenza 2000) to meet energy demand when oxidative phosphorylation is severely diminished. In addition, hypoxia induced genes are involved in cell proliferation/survival, apoptosis, iron-metabolism,

transcriptional regulation, cell adhesion, cytoskeletal structure, nucleotide metabolism, and extracellular matrix metabolism (Semenza 1999; Wenger 2002; Safran and Kaelin 2003; Semenza 2003). Overall, these genes help cells survive under hypoxic conditions.

Hypoxic expression of the EPO gene was shown to be mediated by a 3' *cis*-acting element now known as Hypoxia Response Element (HRE) (Beck, Ramirez et al. 1991; Pugh, Tan et al. 1991; Semenza, Nejfelt et al. 1991). Many of the hypoxia inducible genes were found to contain similar HREs and sequence analysis revealed that HREs are composed of 5'-RCGTG-3' (R is A or G) core consensus sequence (Wenger, Stiehl et al. 2005). Understanding of the relation between HRE and hypoxic induction of gene expression came from identification of a nuclear factor designated as Hypoxia Inducible Factor 1 (HIF-1) (Semenza and Wang 1992). HIF-1 bound to EPO HRE in a sequence-specific manner and it was present only in nuclear extracts of hypoxia treated cells but not in normoxic nuclear extracts. HIF-1 was later affinity purified from a nuclear extract of hypoxic HeLa cells (Wang and Semenza 1995).

HIF-1 is a heterodimeric transcription factor composed of α- and β-subunits (Wang, Jiang et al. 1995). The β-subunit is identical to Arylhydrocarbon Receptor Nuclear Translocator (ARNT) (Wang, Jiang et al. 1995), which was initially identified as dimerization partner of ligand-activated Arylhydrocarbon Receptor (AhR) transcription factor (Hoffman, Reyes et al. 1991). HIF-1β mRNA is ubiquitously expressed (Wenger, Rolfs et al. 1996; Wiener, Booth et al. 1996) and though protein levels are practically insensitive to changes in oxygen level, translocation of ARNT from cytoplasm to nucleus had been observed under hypoxic conditions (Wang, Jiang et al. 1995). On the other hand,

despite the ubiquitous expression of its mRNA (Wenger, Rolfs et al. 1996; Wiener, Booth et al. 1996), HIF-1α protein level is sensitive to oxygen concentration, that is, detectable under hypoxic (<5% O_2) but not normoxic conditions (~20% O_2) (Jiang, Semenza et al. 1996). HIF-1α was also independently identified as ARNT- (Li, Ko et al. 1996) and p300/CBP-interacting protein (Arany, Huang et al. 1996). Later, it was shown that two other genes encode for HIF α-subunits, HIF-2α (also known as Endothelial PAS domain protein 1; EPAS-1) (Ema, Taya et al. 1997; Flamme, Frohlich et al. 1997; Hogenesch, Chan et al. 1997; Tian, McKnight et al. 1997) and HIF-3α (Gu, Moran et al. 1998), both of which are regulated by changes in oxygen level similar to HIF-1α (Wiesener, Turley et al. 1998; O'Rourke, Tian et al. 1999; Srinivas, Zhang et al. 1999). However, neither their tissue expression pattern (Tian, McKnight et al. 1997; Gu, Moran et al. 1998; Wiesener, Jurgensen et al. 2003) nor target gene specificity completely overlaps with that of HIF-1α (Hu, Wang et al. 2003; Sowter, Raval et al. 2003). The role of HIF-3α in hypoxia inducible gene expression is unclear. Interestingly, a shorter splice variant of HIF-3α, which has bHLH-PAS domains but lacks transactivation domain, has been cloned and implicated as a HIF α-subunit antagonist thus named Inhibitory PAS (IPAS) (Makino, Cao et al. 2001).

HIF-1α and HIF-1β are basic-helix-loop-helix-PAS domain (Wang, Jiang et al. 1995) (bHLH-PAS, PAS acronym for PER, ARNT, and SIM, the first proteins identified that contain this domain (Gu, Hogenesch et al. 2000)) transcription factors (Fig. 1.1A). bHLH-PAS domains mediate α- and β-subunit dimerization and DNA-binding (Jiang, Rue et al. 1996; Pugh, O'Rourke et al. 1997). Although PAS domains of HIF subunits have also been

implicated in HIF activation by binding to Heat Shock Protein 90 (HSP90) chaperone (Minet, Mottet et al. 1999), a more careful recent study refutes such a function of these domains (Yang, Zhang et al. 2005). In addition, the α-subunit contains two transcriptional activation domains, N-terminal (NTAD) and C-terminal Transactivation Domains (CTAD), whereby it interacts and recruits transcriptional coactivators and transcriptional machinery to induce target gene expression (Jiang, Rue et al. 1996; Jiang, Zheng et al. 1997; Pugh, O'Rourke et al. 1997).

Regulation of HIF by α-subunit stability: Prolyl hydroxylation

The current understanding of oxygen-dependent regulation of HIF was greatly facilitated by the discovery of ubiquitin-mediated proteosomal degradation of its α-subunit (Huang, Gu et al. 1998; Kallio, Wilson et al. 1999) (Fig. 1.1B). Deletion analysis revealed that a region of HIF-1α, which overlaps with NTAD, confines oxygen-sensitive degradation and was thus named the Oxygen-dependent Degradation Domain (ODD) (Huang, Gu et al. 1998). Removal of the ODD led to stabilization of HIF-1α under normoxia while it triggered oxygen-dependent degradation when fused to an irrelevant protein. Consistent with its negative effect on expression of hypoxia inducible genes (Iliopoulos, Levy et al. 1996), the product of von Hippel-Lindau tumor suppressor gene (pVHL), specificity factor of pVHL/Elongin B/Elongin C E3 ubiquitin ligase complex, was shown to mark HIF α-subunit for proteosomal degradation under normoxic condition (Maxwell, Wiesener et al. 1999; Cockman, Masson et al. 2000; Kamura, Sato et al. 2000; Ohh, Park et al. 2000; Tanimoto, Makino et al. 2000). A major breakthrough came with the discovery that recognition by

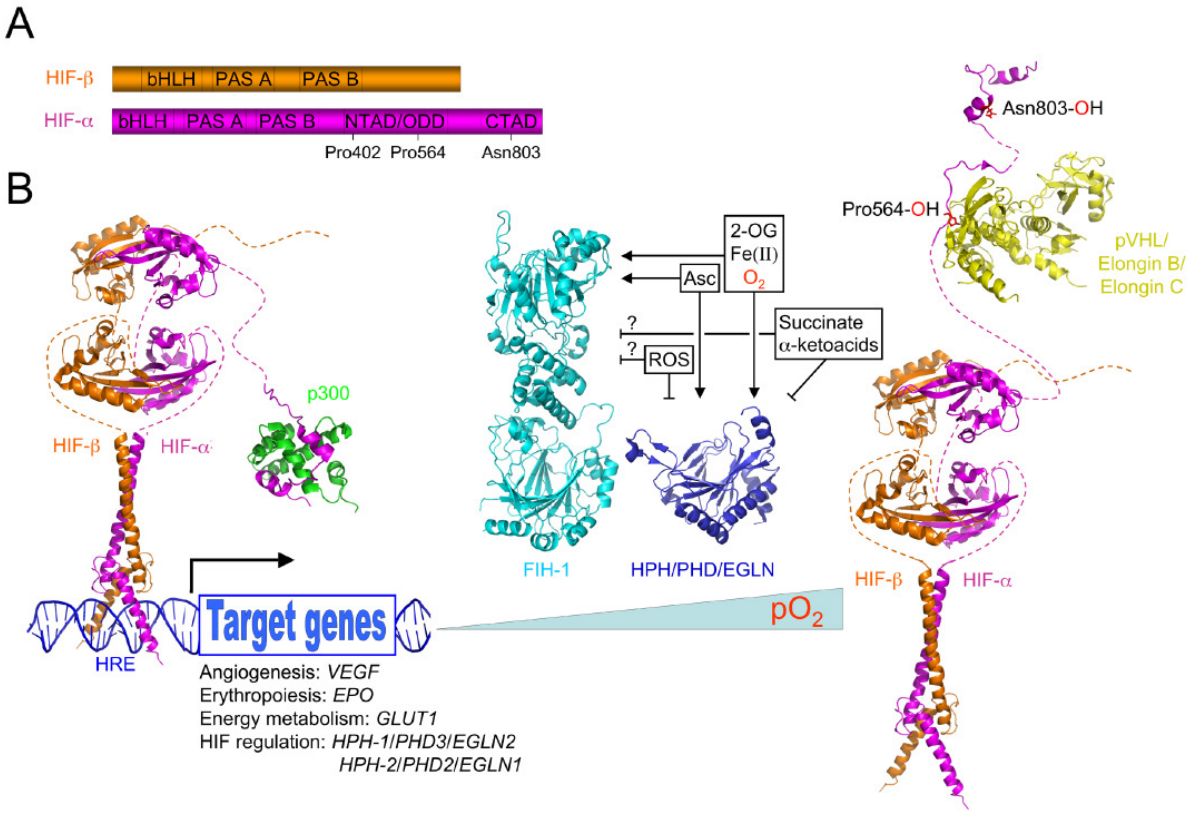


Figure 1.1 Regulation of the mammalian hypoxia response pathway.
(**A**) Domain organization of HIF α- and β-subunits.
(**B**) HIF regulation by Fe(II)- and 2-oxoglutarate-dependent dioxygenases. Under hypoxic conditions, the HIF heterodimer binds to HIF Response Elements (HRE) in the promoter regions of its target genes and recruits transcriptional coactivators such as p300 (CH1 domain shown in green) via the CTAD. Under normoxic conditions, the HIF-α subunit is hydroxylated by prolyl (HPH/PHD/EGLN) and asparaginyl (FIH-1) hydroxylases to promote recruitment of a ubiquitin ligase complex containing pVHL (yellow) or to block coactivator recruitment, respectively. Activities of the dioxygenases are regulated not only by the availability of oxygen, 2-oxoglutarate, and iron, but are also sensitive to oxidative stress (ROS), metabolite concentrations (succinate, α-ketoacids, ascorbate), and regulatory feedback loops. Representative structures were derived from PDB ID: 2A24; 1L3E; 2HBT; 1MZF; 1LM8. The structure of the MAX bHLH homodimer bound to DNA (PDB ID: 1AN2) was used to represent HIF-α and -β bHLH dimerization and DNA binding. Structure figures were generated with PyMOL program. (Figures and figure legends, except for Figure 1.3, are taken from (Ozer and Bruick 2007)).

pVHL is mediated by oxygen-dependent proline hydroxylation of HIF-1α ODD, which is diminished under hypoxic conditions (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001; Yu, White et al. 2001). In addition to the primary hydroxylation site (Pro564 of human HIF-1α) a second proline residue (Pro402) was later found to be hydroxylated and recognized by pVHL (Masson, Willam et al. 2001). X-ray crystallographic studies provided detailed information about how pVHL discriminates hydroxyproline from proline (Hon, Wilson et al. 2002; Min, Yang et al. 2002). Hydroxyproline makes two critical hydrogen bond contacts with pVHL residues; Ser111 and His115 (Fig. 1.2). In the absence of proline hydroxylation (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001; Yu, White et al. 2001) or active pVHL complex (Iliopoulos, Levy et al. 1996; Maxwell, Wiesener et al. 1999; Cockman, Masson et al. 2000; Kamura, Sato et al. 2000; Ohh, Park et al. 2000; Tanimoto, Makino et al. 2000), HIF-1α escapes degradation and accumulates in nucleus where it heterodimerizes with HIF-1β and induce expression of HIF target genes.

Fe(II), 2-oxoglutarate, and oxygen dependence of HIF-1α hydroxylation implicated the presence of proline hydroxylase(s), similar to collagen modifying prolyl hydroxylase (Hutton, Trappel et al. 1966), in the regulation of HIF-1α stability. HIF prolyl hydroxylases were later independently identified by three groups and alternatively named HPH-1/PHD3/EGLN2, HPH-2/PHD2/EGLN1, and HPH-3/PHD1/EGLN3 (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001; Ivan, Haberberger et al. 2002) (which will be referred as HPH-1, HPH-2, and HPH-3 from hereon). The use of oxygen during the hydroxylation reaction suggested a direct link between oxygen concentration and control of HIF-α stability.

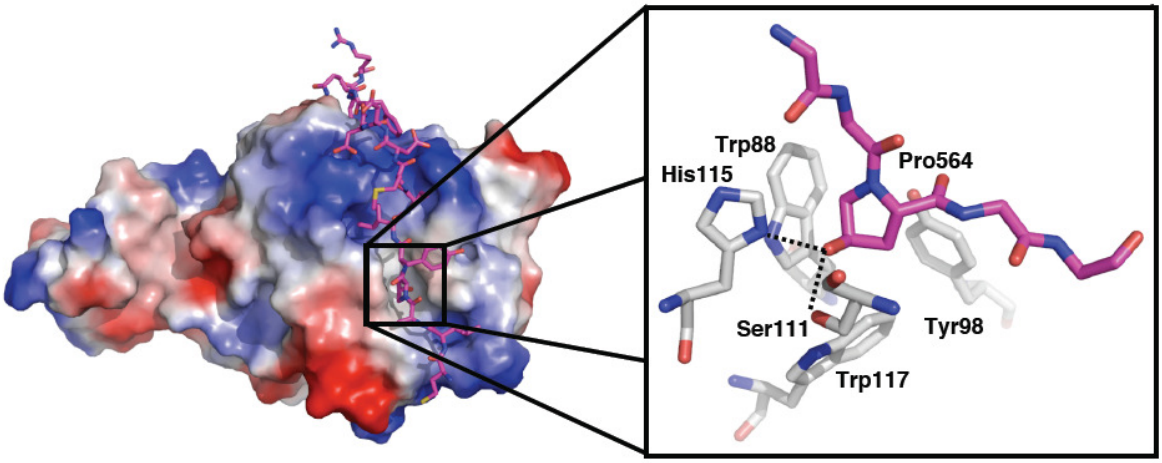


Figure 1.2 Regulation of pVHL-HIF-α interaction by hydroxylation.
Binding of a hydroxylated peptide derived from the HIF-1α ODD (magenta) to pVHL (PDB ID: 1LM8) (Hon, Wilson et al. 2002; Min, Yang et al. 2002). Key hydrogen bond contacts between pVHL and the hydroxylated proline are indicated on the right. The surface representation of pVHL is colored to reflect electrostatic charge (blue = positively charged amino acid side chains, red = negatively charged amino acid side chains).

All three HPH enzymes, which are capable of destabilizing HIF-1α when overexpressed, share a similar C-terminal domain responsible for hydroxylase activity (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001) (Fig. 1.3). However, the N-terminal region of HPH enzymes, which HPH-1 does not possess, differ significantly. Therefore, they are believed to be involved in enzyme-specific, yet unknown, functions. Unlike HPH-3, the N-terminal region of HPH-2 contains a MYND-type zinc finger (Epstein, Gleadle et al. 2001; Choi, Lee et al. 2005). Interestingly, in addition to regulating the stability of HIF-1α, HPH-2 was found to inhibit transcriptional activity of HIF-1α NTAD but not CTAD (To and Huang 2005).

Furthermore, expression patterns (Lieb, Menzies et al. 2002; Soilleux, Turley et al. 2005), subcellular localizations (Metzen, Berchner-Pfannschmidt et al. 2003; Soilleux, Turley et al. 2005), and substrate specificities (Hirsila, Koivunen et al. 2003) of these enzymes are different. HPH enzymes are co-expressed in multiple tissues (i.e. liver and kidney), however; expression levels of individual enzymes change between tissues in a unique pattern (Lieb, Menzies et al. 2002; Soilleux, Turley et al. 2005). For example, HPH-1 mRNA is expressed at the highest level in the heart while the highest expression of HPH-3 mRNA is observed in testis, where HPH-1 and HPH-2 mRNAs are not even detectable (Lieb, Menzies et al. 2002). Studies involving GFP-fusion proteins revealed that HPH-1 was equally distributed between the nucleus and cytoplasm of the cell, while HPH-2 was predominantly cytoplasmic and HPH-3 was exclusively nuclear (Metzen, Berchner-Pfannschmidt et al. 2003). However, immunohistochemical analysis indicates that all three HPH enzymes are primarily localized to the cytoplasm while weak and strong nuclear

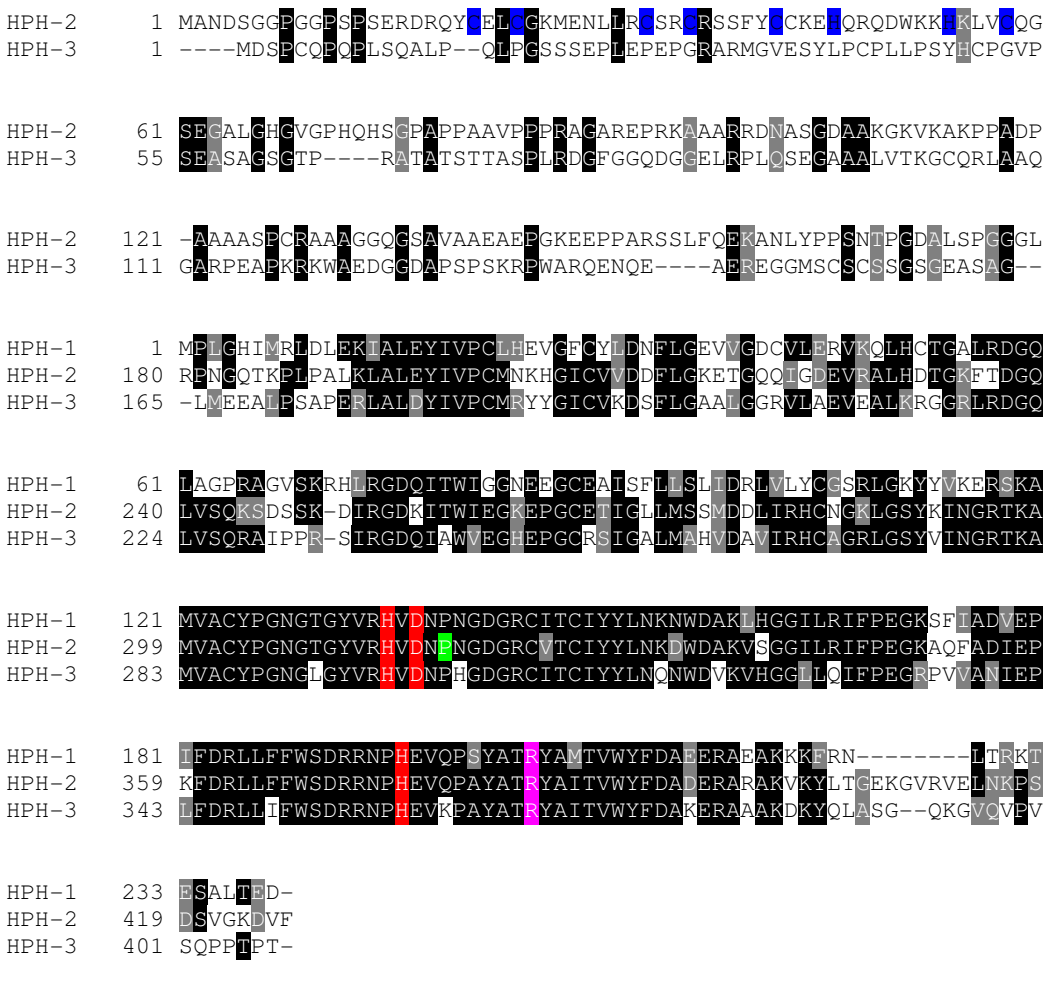


Figure 1.3 Sequence alignment of human HIF prolyl hydroxylases.
Identical residues are shaded in black while similar residues in gray. Catalytic triad (HXD…H motif) is indicated by red, 2-oxoglutarate coordinating arginine residue in purple, site of HPH-2 mutation (Pro317Arg) linked with erythrocytosis is in green, and cysteine and histidine residues forming MNYD-type zinc finger are shown in blue. HPH-1, HPH-2, and HPH-3 sequences were from GeneBank accession #: NP_071356, NP_071334, and NP_444274, respectively. Sequence alignments were done by ClustalW program and shading was done by BoxShade program.

localization of HPH-2 and HPH-1, respectively, was observed in certain tissues (Soilleux, Turley et al. 2005). Furthermore, of the two proline target sites within the LXXLAP hydroxylation motifs of HIF-1α ODD (Masson, Willam et al. 2001), both Pro564 and Pro402 can be hydroxylated by HPH-2 and HPH-3 enzymes, however; HPH-1 is only able to hydroxylate Pro564 *in vitro* (Hirsila, Koivunen et al. 2003).

Moreover, differences in physiological functions of HPH enzymes are emerging as well. In cell culture, siRNA silencing of HPH-2 resulted in HIF-1α stabilization under normoxic conditions while HIF-1α remained undetectable when HPH-1 or HPH-3 were knocked down (Berra, Benizri et al. 2003). This data suggests that HPH-2 is the primary HIF prolyl hydroxylase responsible for targeting HIF α-subunit to proteosomal degradation under normoxic conditions. Conversely, HPH-1 and HPH-3 have been implicated in the regulation of HIF α-subunit stability under hypoxia (Nakayama, Frew et al. 2004). In mice, targeted disruption of individual HPH genes revealed that HPH-2 is required for normal development, HPH-2 knockout embryos die between embryonic day 12.5 and 14.5, whereas HPH-1 and HPH-3 knockout mice are free of any observable developmental defects (Takeda, Ho et al. 2006). Further features of the HPH-2 knockout phenotype include an expected HIF-1α protein upregulation as well as placental and cardiac defects. Surprisingly, in cardiac tissue, disruption of HPH-2 was not accompanied with an expected increase in HIF-1α level suggesting a heart specific HIF-independent function for HPH-2. Even though HPH-1 and HPH-3 knockout mice described in the aforementioned study develop normally, other lines of HPH-1 and HPH-3 knockout mice feature angiogenic defects different from HPH-2

knockout animals (Giaccia, Simon et al. 2004). Therefore, a careful study of these animals will likely yield specific functions of these enzymes in near future.

Regulation of HIF transcriptional activity: Asparaginyl hydroxylation

Soon after the discovery of HIF-1α prolyl hydroxylation, transcriptional activity of HIF was shown to be regulated by yet another oxygen-dependent hydroxylation (Lando, Peet et al. 2002; Sang, Fang et al. 2002). Hydroxylation of an asparagine residue (Asn803 of human HIF-1α) within the CTAD blocks binding of CBP/p300 (Lando, Peet et al. 2002; Sang, Fang et al. 2002) explaining the hypoxia-specific interaction between the two (Ema, Hirota et al. 1999; Carrero, Okamoto et al. 2000; Kung, Wang et al. 2000; Gu, Milligan et al. 2001). The enzyme responsible for this post-translational modification turned out to be Factor Inhibiting HIF 1 (FIH-1) (Hewitson, McNeill et al. 2002; Lando, Peet et al. 2002), which had been previously identified as CTAD interacting protein that repressed HIF transcriptional activity (Mahon, Hirota et al. 2001). Analysis of the hydroxylation reaction revealed that FIH-1 hydroxylates the β-carbon of the asparagine side-chain (McNeill, Hewitson et al. 2002). HIF-1α CTAD, which alone is unstructured in solution, adopts an α-helical structure when bound to CH-1 domain of p300 (Dames, Martinez-Yamout et al. 2002; Freedman, Sun et al. 2002). Within this complex, the target asparagine residue is buried in between the two domains where β-hydroxyasparagine cannot physically fit because of steric hindrance of the two p300 residues, Arg335 and Ile338, explaining how asparagine hydroxylation blocks recruitment of p300 by HIF-1α CTAD (Fig. 1.4).

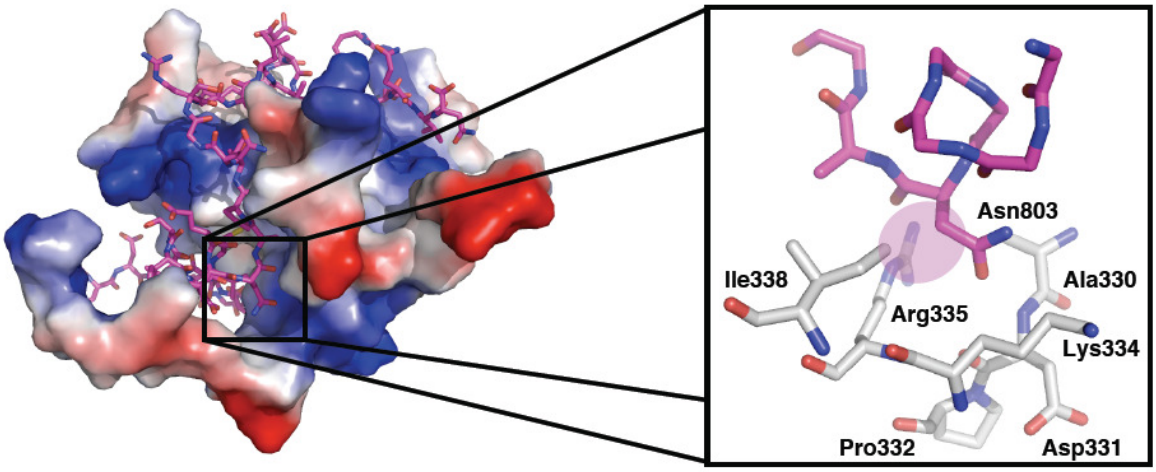


Figure 1.4 Regulation of p300-HIF-α interaction by hydroxylation.
Binding of the HIF-1α CTAD (magenta) to the CH1 domain of p300 (PDB ID: 1L3E)(Dames, Martinez-Yamout et al. 2002; Freedman, Sun et al. 2002). The β-carbon of the Asn803 residue where hydroxylation occurs is highlighted (magenta oval). The surface representation of p300 is colored to reflect electrostatic charge (blue = positively charged amino acid side chains, red = negatively charged amino acid side chains).

FIH-1 is predominantly localized in cytoplasm as determined by fluorescence microscopy of GFP-fusion protein (Metzen, Berchner-Pfannschmidt et al. 2003) as well as immunostaining of endogenous protein (Linke, Stojkoski et al. 2004; Soilleux, Turley et al. 2005). Unlike the HPH enzymes, FIH-1 is almost ubiquitously expressed throughout the body, even in tissues/cells (i.e. kidney glomeruli, lymphocytes, and macrophages) where HPH enzymes are undetectable (Soilleux, Turley et al. 2005). FIH-1 is found to be nuclear in certain tissues though the underlying mechanism and biological consequences of this translocation are unknown (Soilleux, Turley et al. 2005). A FIH-1 knockout mouse has not been reported yet, however; siRNA silencing provides insight about physiological functions of FIH-1. Silencing of FIH-1 caused upregulation of a subset of HIF-target genes under mild (3% O_2) to severe hypoxia (0.2% O_2) while it did not affect them under normoxic conditions (Dayan, Roux et al. 2006). Silencing of HPH-2 is sufficient to stabilize HIF-1α under normoxia (Berra, Benizri et al. 2003), however; it is unable to induce expression of HIF target genes to the same extent as does hypoxia, unless FIH-1 is co-silenced (Dayan, Roux et al. 2006). Taken together, these data suggest that FIH-1 contributes to the regulation of HIF activity in a cell and gene specific manner, even under hypoxic conditions.

Biochemical and structural aspects of HIF prolyl and asparaginyl hydroxylases

HIF prolyl and asparaginyl hydroxylases belong to the Fe(II)- and 2-oxoglutarate-dependent dioxygenase family (Clifton, McDonough et al. 2006; Ozer and Bruick 2007). Members of this protein family are widely distributed in different kingdoms of life and they catalyze a plethora of oxidation reactions modifying substrates ranging from small molecules

to proteins (Clifton, McDonough et al. 2006). Fe(II)- and 2-oxoglutarate-dependent dioxygenases require iron cofactor in its ferrous state (Fe(II)), in addition to the co-substrates 2-oxoglutarate and oxygen (O_2). Iron is coordinated in a hexadentate manner (Fig. 1.5) where three of the coordination sites are occupied by histidine, aspartate/glutamate, and histidine catalytic residues of the enzyme (HXD/E…H motif). Two additional sites are coordinated by 2-oxoglutarate co-substrate (C2 keto and C1 carboxyl groups), and the final site is occupied alternatively by a water molecule or oxygen. This class of dioxygenases utilize both atoms of oxygen for oxidation; one for the prime substrate and one for the 2-oxoglutarate co-substrate (Bugg 2003; Dann and Bruick 2005; Bernhardt 2006; Ozer and Bruick 2007), thus they differ from monooxygenases which utilize one of the two oxygen atoms to oxidize their substrate and the second one is reduced to H_2O with hydrogens donated by reducing agents like $NADPH_2$ (Bernhardt 2006).

All structurally characterized Fe(II) and 2-oxoglutarate-dependent dioxygenases contain a core "beta-jellyroll" domain composed of eight β-strands, forming two sheets of four β-strands. The catalytic core of these enzymes containing 2-oxoglutarate, Fe(II), and Fe(II)-binding HXD/E…H motif, is sandwiched between the two β-sheets. The 2-oxoglutarate co-substrate is almost invariably coordinated by a basic residue, lysine or arginine, again in between the two β-sheets (Dann and Bruick 2005; Clifton, McDonough et al. 2006; Ozer and Bruick 2007).

Reactions catalyzed by Fe(II)- and 2-oxoglutarate-dependent dioxygenases are believed to proceed through a radical mechanism involving an iron-oxo intermediate (Bugg 2003; Hausinger 2004; Dann and Bruick 2005). (Fig. 1.5) A quaternary complex formed

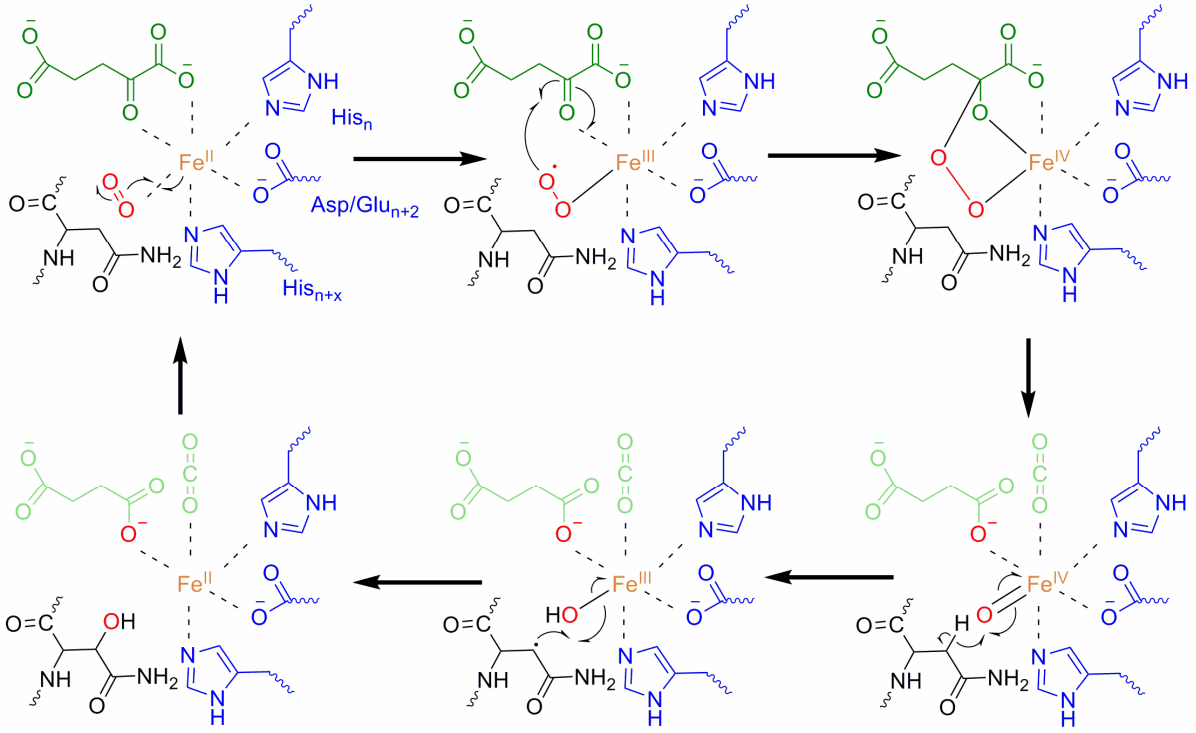


Figure 1.5 Proposed mechanism of oxidation reactions catalyzed by Fe(II)- and 2-oxoglutarate-dependent dioxygenases.

As shown for asparaginyl hydroxylation catalyzed by FIH-1, the reaction proceeds through a radical mechanism involving an iron-oxo intermediate. Amino acid side-chains of the enzyme are colored blue, the prime substrate asparagine is black, the 2-oxoglutarate co-substrate is dark green, while the succinnate and CO_2 products are light green, with the oxygen atoms derived from molecular oxygen (O_2) shown in red. Chemical structures were drawn with ChemDraw program. (Figure and figure legend are taken from (Ozer and Bruick 2007)).

between the enzyme and Fe(II), 2-oxoglutarate, and the prime substrate is activated by incoming oxygen (O_2) which displaces the Fe(II)-bound water molecule. Electron transfer from Fe(II) generates a superoxide radical which attacks C2 of 2-oxoglutarate forming a covalent linkage between the Fe(III) center and 2-oxoglutarate co-substrate. Decarboxylation of 2-oxoglutarate to succinate and carbon dioxide results in formation of a Fe(IV)-oxo intermediate. The Fe(IV)-oxo intermediate is then reduced by hydrogen atom abstracted from the C-H bond at the target site of prime substrate generating a substrate radical. Finally, the hydroxide radical attached to Fe(III) is transferred to the substrate forming the hydroxylated product and regenerating the active Fe(II) center. Overall, the reaction results in transfer of one oxygen atom to the succinate by-product and one to the prime substrate (Bugg 2003; Hausinger 2004; Dann and Bruick 2005). Ascorbate, even though not consumed during the reaction, is required for the full activity of this class of enzymes perhaps for maintenance of the ferrous state of catalytic iron (Myllyharju 2003; Dann and Bruick 2005; Clifton, McDonough et al. 2006).

Recently solved crystal structures of FIH-1 (Dann, Bruick et al. 2002; Elkins, Hewitson et al. 2003; Lee, Kim et al. 2003) and the prolyl hydroxylase domain (amino acids 181 to 426) of HPH-2 (McDonough, Li et al. 2006) are in good agreement with other Fe(II)- and 2-oxoglutarate-dependent dioxygenase enzyme structures (Clifton, McDonough et al. 2006). FIH-1 and the HPH-2 prolyl hydroxylase domain have the typical beta-jellyroll topology (Fig. 1.6). Conserved iron binding HXD…H motif consists of His313, Asp315, and His374 of HPH-2, and His199, Asp201, and His279 of FIH-1. Additionally, the structures of FIH complexed with and without substrate peptide and 2-oxoglutarate have been solved

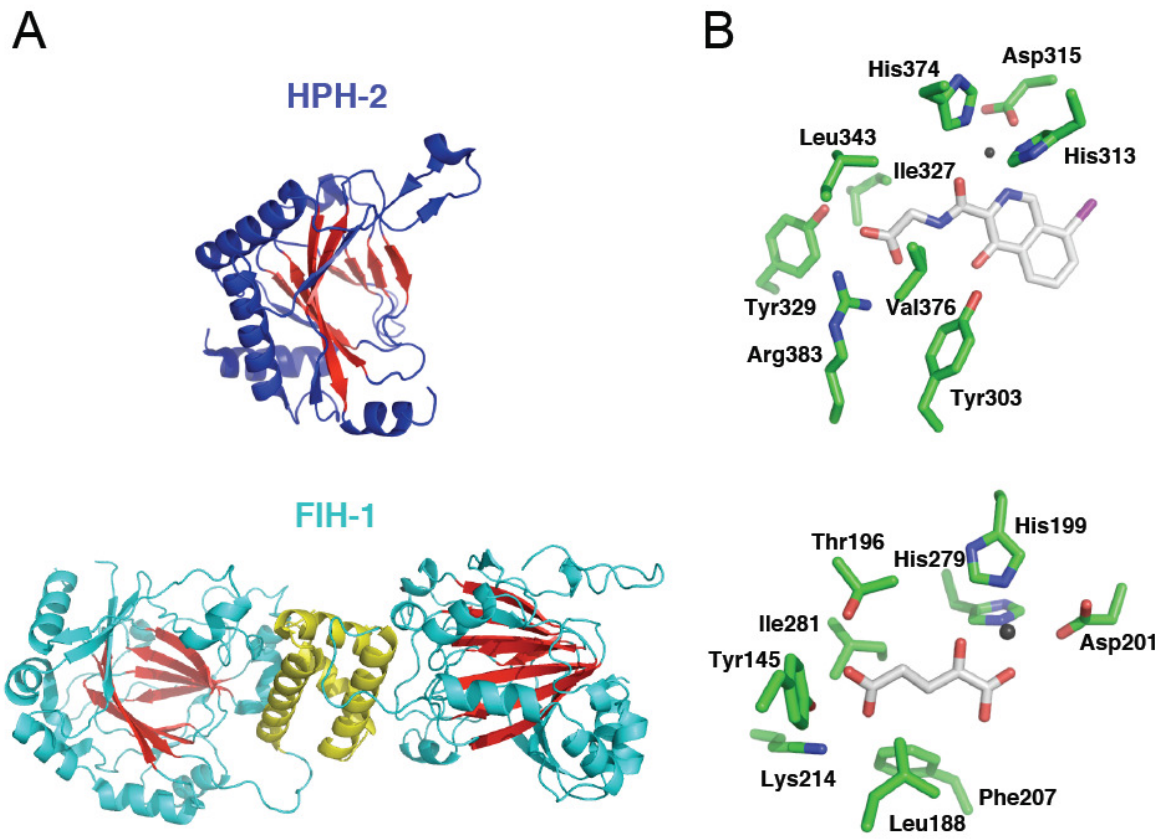


Figure 1.6 Structures of HIF prolyl (HPH-2) and asparaginyl (FIH-1) hydroxylases.
(**A**) Ribbon diagrams representing the structures of HIF hydroxylases (Dann, Bruick et al. 2002; Elkins, Hewitson et al. 2003; Lee, Kim et al. 2003; McDonough, Li et al. 2006). The jellyroll motifs are shown in red and the dimerization domain of FIH-1 is in yellow.
(**B**) The active sites of the HIF hydroxylases feature residues required for Fe(II) (black sphere) and 2-oxoglutarate (gray) binding. The catalytic domain of HPH-2/PHD2/EGLN1 was crystallized with an inhibitor that occupies the 2-oxoglutarate binding site, [(4-hydroxy-8-iodoisoquinolin-3-yl)carbonyl]amino. Figures were generated from PDB ID: 1MZF and 2G19 with PyMOL program.

(Dann, Bruick et al. 2002; Elkins, Hewitson et al. 2003; Lee, Kim et al. 2003) while the only published HPH-2 structure lacks the prime substrate and 2-oxoglutarate, but instead contains a 2-oxoglutarate analog inhibitor (McDonough, Li et al. 2006). 2-oxoglutarate is coordinated by a lysine residue (Lys214) in FIH-1 while an arginine residue (Arg383) is oriented in a similar position in HPH-2 structure. Apart from this, similar hydrophobic and hydrogen-bond interactions are expected between 2-oxoglutarate and residues of FIH-1 or HPH-2. Closer organization of the two β-sheets in HPH-2, compared to FIH-1 (Koivunen, Hirsila et al. 2004), is consistent with its tighter Fe(II) and 2-oxoglutarate binding (Hirsila, Koivunen et al. 2005; McNeill, Flashman et al. 2005). Surprisingly, the C-terminal α-helices in FIH-1 and HPH-2 mediate dimerization (Dann, Bruick et al. 2002; Elkins, Hewitson et al. 2003; Lee, Kim et al. 2003) and trimerization (McDonough, Li et al. 2006), respectively. The dimeric structure of FIH-1 is crucial for its activity (Dann, Bruick et al. 2002; Lancaster, McNeill et al. 2004); however, the functional relevance of HPH-2 trimerization is unclear. While the structural information about the HIF-1α-HPH-2 interaction is lacking, the structure of a FIH-1-HIF-1α CTAD peptide complex is available and indicates a bipartite interaction between the peptide and FIH-1 (Elkins, Hewitson et al. 2003). Interactions of HIF-1α amino acids 795-806 with FIH-1 are stabilized by hydrogen bonds whereas interactions of the second site (residues 813-822) are primarily hydrophobic in nature. HIF-1α residues 795-806 form an α-helix when bound to CH1 domain of p300 (Dames, Martinez-Yamout et al. 2002; Freedman, Sun et al. 2002). However, these residues go through a conformational change when binding FIH-1 and adopt an extended conformation thereby allowing insertion of the hydroxylation target (Asn803) into the active site of FIH-1 (Elkins, Hewitson et al. 2003). On the other

hand, residues of the second site adapt a similar α-helical structure when bound to FIH-1 or p300, restricting interaction of HIF-1α CTAD to one or the other.

Biochemical characterization of HIF hydroxylases were mostly carried out with recombinant proteins and model peptide substrates derived from the C-terminal prolyl hydroxylation site (Pro564) in ODD and the asparaginyl hydroxylation site (Asn803) in CTAD of HIF-1α. K_m values of the three HPH enzymes for the C-terminal peptide substrate are in low micromolar concentrations, 7-8 μM (Hirsila, Koivunen et al. 2003) (Table 1.1). Peptides from N-terminal hydroxylation site (Pro402) or shorter peptides of the C-terminal site are poor substrates (Hirsila, Koivunen et al. 2003), whereas complete HIF-1α ODD (residues 400-600) is a better substrate of HPH enzymes (0.01-0.14 μM K_m value) (Koivunen, Hirsila et al. 2006). Interestingly, hydroxylation at the C-terminal site (Pro564) appears to promote hydroxylation at the N-terminal site (Pro402) (Chan, Sutphin et al. 2005). *In vivo*, hydroxylation of Pro564 precedes Pro402 hydroxylation and, when Pro564 is mutated, Pro402 hydroxylation is attenuated (Chan, Sutphin et al. 2005). On the other hand, FIH-1 requires longer peptide substrates (35 residues) compared to HPH enzymes (19 residues), and even then its K_m value for the peptide substrate is approximately 100 μM (Koivunen, Hirsila et al. 2004). Mutations within these peptide substrates, except for the target proline and asparagine, are tolerable *in vitro* (Huang, Zhao et al. 2002; Linke, Stojkoski et al. 2004). These and other observations (Mahon, Hirota et al. 2001) suggest that the enzyme-substrate interactions of HPH-2 and FIH-1 require multiple contacts some of which are far from the hydroxylation site, and perhaps this extended interaction determines substrate specificity.

Table 1.1 K_m values for HIF modifying Fe(II)- and 2-oxoglutarate-dependent dioxygenases.

	K_m values (μM)			
	HPH-1	**HPH-2**	**HPH-3**	**FIH**
HIF-1α ODD	0.01 - 0.02[a]	0.14 ± 0.02[a]	0.07 ± 0.04[a]	NA
HIF-1α C-TAD	NA	NA	NA	100 ± 5[b]
Oxygen	230[c]	100[a] / 250[c]	230[c]	90 ± 20[b]
2-oxoglutarate	60[c]	<2[e] / 60[c]	55[c]	25 ± 3[b]
Fe(II)	0.03 ± 0.002[d]	0.03 ± 0.004[d]	0.1 ± 0.04[d]	0.5 ± 0.2[b]
Ascorbate	170[c]	180[c]	140[c]	260 ± 50[b]

NA, Not applicable; [a] See (Koivunen, Hirsila et al. 2006); [b] See (Koivunen, Hirsila et al. 2004); [c] See (Hirsila, Koivunen et al. 2003); [d] See (Hirsila, Koivunen et al. 2005); [e] See (McNeill, Flashman et al. 2005); [f] See (Myllyharju and Kivirikko 1997).

HIF prolyl and asparaginyl hydroxylases are known to be sensitive to oxygen concentrations within the physiological range of hypoxia. *In vitro*, the K_m values of all three recombinant HPH enzymes for oxygen are in 230-250 μM range (Hirsila, Koivunen et al. 2003). Closer K_m values of HPH enzymes to that of dissolved oxygen concentration under hypoxic conditions (~200 μM) imply that a slight change in oxygen level will cause a marked reduction in HPH activity, which fulfills the criteria of a good oxygen sensor. On the other hand, inhibition of FIH activity requires a more severe drop in oxygen concentration since K_m value of FIH for oxygen is around 100 μM (Koivunen, Hirsila et al. 2004). This allows the regulation of HIF to be made precisely with two regulators, HPHs and FIH-1, tuned at different oxygen concentrations. Indeed, it has been shown that *in vivo*, FIH-1 remains active at low oxygen concentrations (>0.5 % O_2) at which HIF prolyl hydroxylases are inactivated (Stolze, Tian et al. 2004).

HIF hydroxylase enzymes require not only oxygen but also 2-oxoglutarate, Fe(II), and ascorbate. K_m values for each one of these co-substrate and co-factors have been measured as well (Hirsila, Koivunen et al. 2003; Koivunen, Hirsila et al. 2004; McNeill, Flashman et al. 2005). Half-maximal activity of FIH-1 is reached with ~25 μM 2-oxoglutarate *in vitro* (Koivunen, Hirsila et al. 2004) while HPH enzymes require higher concentrations (~60 μM) (Hirsila, Koivunen et al. 2003). However, it should be noted that, using a different enzyme source and assay method, the K_m value of HPH-2 for 2-oxoglutarate has been measured to be less than 2 μM (McNeill, Flashman et al. 2005). In contrast, FIH-1 activity requires a higher concentration of ascorbate (K_m of ~260) (Koivunen, Hirsila et al. 2004) than HPH enzymes (~150 μM) (Hirsila, Koivunen et al. 2003). With regards to iron cofactor, the apparent K_m values of recombinant HPH and FIH-1 enzymes are measured to be in 30-100 nM (Hirsila, Koivunen et al. 2005) and 0.5 μM (Koivunen, Hirsila et al. 2004), respectively.

HIF prolyl and asparaginyl hydroxylases: Sensors of cellular environment?

In vivo, the real K_m value of HPH enzymes for oxygen is likely to be lower than 230-250 μM (Hirsila, Koivunen et al. 2003). When measured *in vitro* using HIF-1α ODD instead of a peptide substrate, the values are around 100 μM (Koivunen, Hirsila et al. 2006), similar to that of FIH-1 (Koivunen, Hirsila et al. 2004). K_m value of 100 μM is significantly lower than dissolved oxygen concentration under hypoxia (~200 μM), however; the effective concentration of oxygen within the cell may be lowered by mitochondrial consumption as such that it allows HPH enzymes and FIH-1 to act as oxygen sensors (Wenger 2006).

Nevertheless, several groups argue that the mitochondria, as opposed to HPHs and FIH-1, are the actual oxygen sensors which in turn regulate HIF hydroxylases in response to changes in oxygen availability (Bell, Emerling et al. 2005). Data supporting this model and its proposed mechanism of HIF and HIF hydroxylase regulation are still controversial.

HIF hydroxylases may also serve as sensors of the intracellular environment, especially metabolic state and oxidative stress by monitoring levels of metabolites and reactive oxygen species, respectively. The 2-oxoglutarate sensory function of HIF hydroxylases seems unlikely since estimates of cellular 2-oxoglutarate concentration (10-50 mM) (Lawson, Guynn et al. 1976) are well above their *in vitro* K_m values. However, it is not clear whether 2-oxoglutarate is equally present in the cytoplasm and nucleus where HIF hydroxylases reside or is it concentrated in mitochondria. Succinate, an intermediate of the mitochondrial Tricarboxylic Acid (TCA) cycle like 2-oxoglutarate, is produced by decarboxylation of the 2-oxoglutarate co-substrate in proline and asparagine hydroxylation reactions. Succinate inhibits HPH enzymes with an IC_{50} value of 0.5 mM *in vitro* (Selak, Armour et al. 2005). *In vivo*, such a high concentration has been measured with inhibition of Succinate Dehydrogenase (SDH) enzyme (Selak, Armour et al. 2005) responsible for converting succinnate to fumarate. Likewise, fumarate and related α-ketoacids have been shown to inhibit HIF hydroxylases (Dalgard, Lu et al. 2004; Lu, Dalgard et al. 2005; Selak, Armour et al. 2005).

Ascorbate, required for the full activity of HIF hydroxylases, is believed to help maintain the ferrous state (Fe(II)) of iron that is bound to the enzyme in the resting state (Clifton, McDonough et al. 2006). Physiological levels of ascorbate (25-50 μM) are well

below the K_m values of HIF prolyl (~150 μM) and asparaginyl hydroxylases (~250 μM) (Knowles, Raval et al. 2003). Therefore, changes in ascorbate levels are likely to be translated by HIF hydroxylases *in vivo*. Oxidative stress/reactive oxygen species (ROS) can promote oxidation of ferrous iron to ferric iron (Fe(III)) either directly or indirectly by reducing ascorbate levels. Ferric iron, due to its electronic configuration, does not permit catalysis of hydroxylation and inactivates the HIF hydroxylases. Though HIF hydroxylases are predicted to sense Fe(II)/Fe(III) ratio as a measure of cellular oxidative stress, it is unlikely that they function as direct iron sensors under physiological conditions since bioavailable iron (5-15 μM) (Kruszewski 2004) is in excess of their K_m values (30-100 nM for HPHs (Hirsila, Koivunen et al. 2005), and 0.5 μM for FIH-1 (Koivunen, Hirsila et al. 2004)). Taken all together, these data suggest a privileged role for HIF hydroxylases as cellular sensors of not only oxygen but also metabolic state and oxidative stress.

Regulation of HIF prolyl and asparaginyl hydroxylases

As for HIF, hydroxylases that modify HIF are subjected to multiple layers of regulation. Physiological oxygen concentrations experienced by most tissue (2-5% O_2) are far from normoxic conditions of cell culture (21% O_2). HIF α-subunit and HIF-1α ODD-luciferase fusion protein are barely detectable within live animal tissues, albeit their significant induction in cells cultured at similarly low oxygen concentrations (Safran, Kim et al. 2006). An explanation for this apparent discrepancy has been offered by a negative feedback loop constituted by HIF and HIF prolyl hydroxylases resetting the threshold for hypoxia (Marxsen, Stengel et al. 2004; Khanna, Roy et al. 2006; Stiehl, Wirthner et al. 2006).

Non-saturating levels of HPH enzymes are sufficient to keep HIF-α proteins at a basal level under normoxic condition. Exposure to hypoxia leads to activation of HIF and upregulation of HPH-1 and HPH-2 enzymes which are among the HIF-target genes (Stiehl, Wirthner et al. 2006). Accumulation of HPH enzymes under hypoxic conditions not only ensures swift destruction of HIF α-subunits upon reoxygenation (half-life of HIF-1α is ~5 min (Wang, Jiang et al. 1995; Jewell, Kvietikova et al. 2001)) but also initiates degradation of HIF α-subunits with the limited oxygen availability (Appelhoff, Tian et al. 2004; Khanna, Roy et al. 2006; Stiehl, Wirthner et al. 2006). Cells adapted to lower oxygen concentrations (i.e. 5% O_2, which is normally sensed as hypoxic) for extended periods of time gradually lose HIF α-subunits and require even lower concentrations of oxygen for stabilization of HIF α-subunits. Conversely, cells adjusted to higher oxygen concentrations (i.e. 30% O_2) reduce expression of HPH enzymes to compensate for their increased activity. Therefore, exposure of the same cells to 21% O_2, which would normally sensed as normoxic, leads to stabilization and activation of HIF. This dynamic feedback loop allows cells to adjust their "normoxic setpoint" to a given oxygen concentration (Appelhoff, Tian et al. 2004; Khanna, Roy et al. 2006; Stiehl, Wirthner et al. 2006).

In addition to the regulatory feedback mechanism, several other regulatory mechanisms of HIF hydroxylases have been discovered recently. The N-terminal region of HPH-2 contains a MYND-type zinc finger (Epstein, Gleadle et al. 2001; Choi, Lee et al. 2005), which has been shown to inhibit hydroxylase activity (Choi, Lee et al. 2005). Lack of similar zinc fingers in other HPH enzymes suggests a unique auto-regulatory mechanism for HPH-2. OS-9 initially identified as a HIF-1α interacting protein was shown to mediate

formation of a HIF-1α/OS-9/HPH ternary complex by strengthening the interaction between HIF-1α and HPH-1 or HPH-2 (Baek, Mahon et al. 2005). It is this strengthened interaction rather than an increase in HPH activity that is the cause of HIF-1α destabilization associated with OS-9 overexpression. Similar modes of regulation, if they exist, have not been discovered for FIH-1. Nonetheless, both HIF prolyl hydroxylases and FIH-1 were found to be subjected to proteosomal degradation in an oxygen-dependent manner.

HPH-1, HPH-3 (Nakayama, Frew et al. 2004), and FIH-1 (Fukuba, Yamashita et al. 2007) are targeted for proteosomal degradation by the RING-finger E3 ubiquitin ligases SIAH1 and SIAH2. Increased protein level and half-life of HPH-3 in SIAH1/2 double-null cells are accompanied by a reduction in HIF-1α stability and HIF target gene expression under hypoxia (Nakayama, Frew et al. 2004). Likewise, FIH-1 protein level is induced by siRNA silencing of SIAH1 and repressed by its overexpression (Fukuba, Yamashita et al. 2007). HPH-2, the master regulator of HIF-1α stability under normoxia (Berra, Benizri et al. 2003), is not affected even though it can be recognized by SIAH2 (Nakayama, Frew et al. 2004). Hypoxic induction of SIAH1 (Fukuba, Yamashita et al. 2007) and SIAH2 (Nakayama, Frew et al. 2004) indicate a hypoxia-specific function for these proteins in regulation of HPH-1, HPH-3, and FIH-1 protein levels and ultimately in regulation of HIF α-subunit stability and transcriptional activity, respectively. Thus, SIAH proteins add another layer of complexity to the hypoxia response pathway.

Correlations between HIF activation and tumor progression/resistance to therapy and HIF's preventive effects against ischemic damage made HIF, and recently HIF hydroxylases, popular targets for therapy (Paul, Simons et al. 2004; Ratan, Siddiq et al. 2004; Belozerov

and Van Meir 2005; Brahimi-Horn and Pouyssegur 2005; Semenza 2006). HPH-2 has been linked with erythrocytosis as members of a family diagnosed with high red blood cell counts were found to carry a mutation in the HPH-2 gene (Percy, Zhao et al. 2006). The observed mutation, Pro317 to Arg, was in close proximity to HPH-2's catalytic site, which includes His313 and Asp315 residues, and was shown to inhibit its hydroxylase activity. Induction of HIF as a consequence of HPH-2 inhibition is thus believed to be the underlying cause of erythrocytosis (Percy, Zhao et al. 2006). Furthermore, a decrease in HPH expression is also observed in several different types of tumors (Soilleux, Turley et al. 2005). Surprisingly, overexpression and nuclear translocation of HPH-2 is associated with tumor aggressiveness in head and neck squamous cell carcinoma (Jokilehto, Rantanen et al. 2006). These observations lay the basis of investigating HIF prolyl hydroxylases as novel therapeutic targets.

In animals, targeted inhibition of HPH enzymes by injection of HPH-2 specific siRNA (Natarajan, Salloum et al. 2006) and administration of the prolyl hydroxylase inhibitor dimethyloxallyl glycine (DMOG) (Milkiewicz, Pugh et al. 2004; Ockaili, Natarajan et al. 2005) showed promising preliminary results in prevention of ischemic injuries of brain and heart. Furthermore, small molecule inhibitors of HPH enzymes (3,4-dihydroxybenzoate and compound A (Fibrogen)) manifested neuroprotective effects against ischemic injury in rats (Siddiq, Ayoub et al. 2005). Amphotericin B (AmB), an anti-fungal agent used for treatment of systemic infections, is known to cause anemia with long-term treatments (Gallis, Drew et al. 1990). AmB-induced anemia is caused by suppression of erythropoietin (EPO) expression, a well known HIF target gene (Yeo, Ryu et al. 2006). Interestingly, AmB has no

effect on HIF-1α stability but inhibits transcriptional activity of CTAD by enhancing FIH-1/CTAD interaction (Yeo, Ryu et al. 2006). Despite these promising observations, pharmacological use of these compounds will be limited because of their inherent non-specific inhibitory effects on other Fe(II)- and 2-oxoglutarate-dependent dioxygenases. However, crystal structures of HPH-2 and FIH-1 will greatly facilitate the design of specific inhibitors, some of which have already emerged (Warshakoon, Wu et al. 2006; Warshakoon, Wu et al. 2006; Warshakoon, Wu et al. 2006; Warshakoon, Wu et al. 2006).

On the other hand, inhibition of HIF activity by means of HPH and/or FIH-1 activation might have a therapeutic value in cancer treatment. A diacylglycerol kinase inhibitor compound (R59949) has been shown to destabilize HIF α-subunit even under hypoxic condition by activating HPH enzymes (Temes, Martin-Puig et al. 2005). Such a molecule might be used to overcome the resistance of HIF-positive tumors to radiotherapy and chemotherapy. Likewise, co-substrates and co-factors of HIF hydroxylases could be used to activate these enzymes. In a proof of principle experiment, 2-oxoglutarate treatment results in HIF-1α degradation in cell culture (Matsumoto, Imagawa et al. 2006).

HIF-independent functions and novel (candidate) substrates of HIF prolyl and asparaginyl hydroxylases

HIF hydroxylases have also been implicated in the regulation of other proteins besides HIF α-subunits (HIF-1α, -2α, and -3α). Both prolines (Pro564 and Pro402 of HIF-1α) that are hydroxylated by HIF prolyl hydroxylases reside in a conserved LXXLAP motif. The largest subunit of RNA polymerase II, RPB1, contains an identical motif (LGQLAP).

Hydroxylation of the proline residue within this motif leads to ubiquitination and subsequent proteosomal degradation of RPB1 mediated by the pVHL complex (Kuznetsova, Meller et al. 2003). The striking similarity between HIF α-subunit and RPB1 regulation suggest a role for HIF prolyl hydroxylases as a modifier of RPB1. Since the conclusive evidence linking HPH enzymes to hydroxylation of RPB1 is missing, it remains possible that yet an unidentified prolyl hydroxylase(s) could modify RPB1.

Like HIF, NF-κB is one of several transcription factors induced by hypoxia (Cummins and Taylor 2005). Presence of LXXLAP motifs on two major regulators of NF-κB, Iκ-B Kinase α and β (IKK-α, IKK-β) led to the investigation of HIF prolyl hydroxylases as a potential link between oxygen concentration and NF-κB activation (Cummins, Berra et al. 2006). NF-κB is normally sequestered in the cytoplasm by Iκ-B and the activation of NF-κB requires dissociation of Iκ-B, which is induced by phosphorylation of Iκ-B by Iκ-B Kinases (IKKs). IKKs are in turn activated by diverse stimuli; however, the mechanism of NF-κB activation in response to hypoxia has remained largely unknown. Effects of HPH-2 and HPH-3 inhibition and overexpression are consistent with a model whereby HIF prolyl hydroxylases inhibit NF-κB activity by hydroxylation-dependent inactivation of IKKs. Therefore, the inhibition of HPH activity under hypoxic conditions in turn activates NF-κB (Cummins, Berra et al. 2006). The mechanism by which this speculative hydroxylation would inactivate IKKs remain unclear since these proteins were neither ubiquitinated nor degraded in response to manipulations of HPH enzymes (Cummins, Berra et al. 2006). Interestingly, ankyrin repeats of Iκ-B and the NF-κB p50 subunit

precursor were found to associate with FIH-1 (Cockman, Lancaster et al. 2006). Unlike the HPH-IKK interaction, interaction of ankyrin repeat proteins with FIH-1 results in their hydroxylation but the biological function of these modifications remain enigmatic. Nevertheless, all together these data suggest multiple functions for HIF hydroxylases in environmental and metabolic sensing and regulation of cellular pathways. Moreover, HIF hydroxylases and related Fe(II)- and 2-oxoglutarate-dependent dioxygenases have been implicated in regulation of several other pathways/proteins including but not limited to cell growth and proliferation (Frei and Edgar 2004), iron-homeostasis (Hanson, Rawlins et al. 2003; Wang, Chen et al. 2004), posttranslational modifications of proteins (Stenflo, Holme et al. 1989; van der Wel, Ercan et al. 2005), and reversal of protein methylation (Ozer and Bruick 2007). Future investigations will no doubt reveal additional biological functions of HIF hydroxylases.

Soon after the identification HIF prolyl hydroxylases (HPH-1, HPH-2, and HPH-3) responsible for regulation of HIF α-subunit stability, Richard K. Bruick and Leeju C. Wu performed a yeast two-hybrid screen to identify HPH-2 interacting proteins with the expectation that such proteins might be novel substrates of HPH-2 or regulators of its activity. To this end, the C-terminal catalytic domain of HPH-2 (HPH-2C) was used as bait and screened against a cDNA library derived from the HBL100 breast mammary gland cell line. Of the 94 positive clones identified; 53 clones were from a single gene called Inhibitor of Growth family member 4 (ING4), 18 from HIF-α subunits (HIF-1α (13) and HIF-2α (5), known substrates of HPH-2 enzyme), and rest of the clones were from separate genes.

In this thesis, I will describe our efforts towards understanding the regulatory mechanisms that govern one of the major components of mammalian hypoxia response pathway, namely HIF prolyl hydroxylases (HPHs). We studied the protein-protein interactions of HPH enzymes, which we identified using several methods. Furthermore, we not only characterized functional consequences of these interactions with respect to HIF activity but also tried to delineate the underlying mechanism of their effects. In Chapter 2, I will describe our effort to characterization of ING4-HPH2 interaction and effect of ING4 on HIF. In Chapter 3, you will find purification of the ING4 protein complex and characterization of this complex's function with respect to HIF and an additional hypoxia inducible transcription factor, NF-κB. In Chapter 4, I will describe identification of additional HIF prolyl and asparaginyl hydroxylase interacting proteins and our efforts to characterize functions of these proteins. Finally in Chapter 5, you will find a summary of the key findings of the previous chapters and their implications for future studies.

CHAPTER TWO
ING4, an HPH-2 binding protein, functions as a HIF repressor

Introduction

The Inhibitor of Growth (ING) protein family is a small family of nuclear proteins that share a highly conserved Plant Homeodomain (PHD) at the C-termini. The founding member of this protein family, ING1, was initially identified in a subtractive hybridization experiment that assessed differentially expressed genes between normal and cancer cells (Garkavtsev, Kazarov et al. 1996). ING1 was then further characterized as a candidate tumor suppressor protein, as its overexpression induced arrest at the G1/G0 transition of the cell cycle and conversely, antisense mRNA mediated suppression of ING1 resulted in cellular transformation (Garkavtsev, Kazarov et al. 1996). Since the identification of ING1 in 1996, four other members of the ING family –ING2 (Shimada, Saito et al. 1998), ING3 (Nagashima, Shiseki et al. 2003), ING4, and ING5 (Shiseki, Nagashima et al. 2003)– have been identified primarily based on sequence similarity with ING1. ING family proteins, including splice variants of ING1, contain identifiable nuclear localization signals (NLS) as well as highly similar C-terminal Plant Homeodomain (PHD)-type zinc fingers (Feng, Hara et al. 2002; Campos, Chin et al. 2004) (Fig. 2.1).

PHD zinc fingers are found almost exclusively in proteins involved in chromatin remodeling and chromatin-dependent transcriptional regulation, which ING proteins are not exceptions (Aasland, Gibson et al. 1995). These domains are 50-100 amino acids in length and contain a conserved $Cys_4HisCys_3$ motif ($Cys\text{-}X_{1\text{-}2}\text{-}Cys\text{-}X_{9\text{–}21}\text{-}Cys\text{-}X_{2\text{–}4}\text{-}Cys\text{-}X_{4\text{-}5}\text{-}His\text{-}X_{2}$-

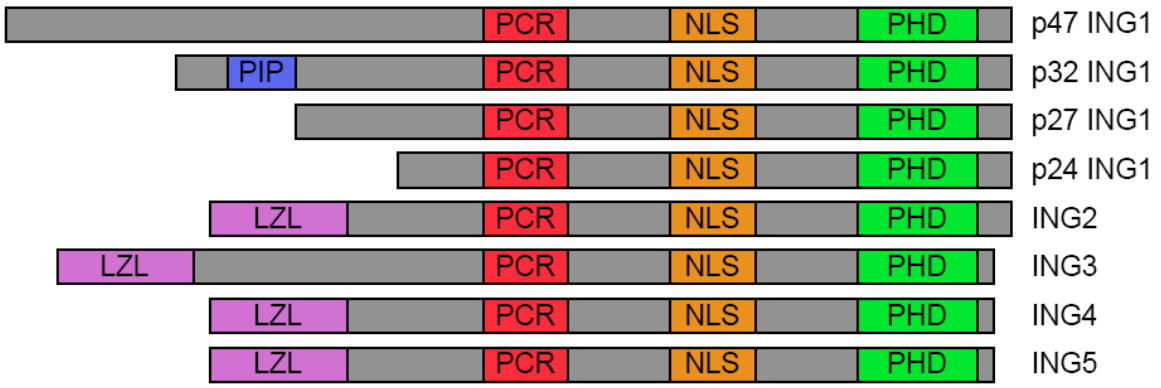


Figure 2.1 Domain organization of Inhibitor of Growth (ING) family proteins in *Homo sapiens*.
Human ING family proteins including isoforms of ING1 are shown in diagram with domains indicated by different color boxes: PIP (blue), proliferating cell nuclear antigen (PCNA)-interacting protein domain; LZL (magenta), leucine zipper-like domain; PCR (red), potential chromatin regulatory domain; NLS (orange), nuclear localization signal; PHD (green), plant homeodomain. Adapted from (He, Helbing et al. 2005).

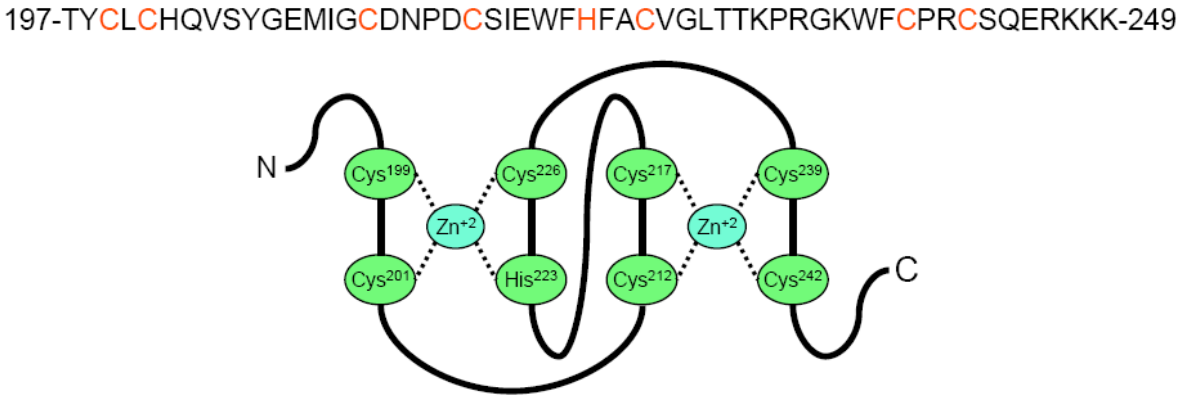


Figure 2.2 Plant Homeodomain (PHD)-type zinc finger.
Amino acid sequence of ING4 PHD zinc finger and coordination of two zinc atoms by conserved $Cys_4HisCys_3$ motif. Cartoon model for PHD zinc finger was adapted from (Kwan, Gell et al. 2003).

Cys-$X_{12–46}$-Cys-X_2-Cys consensus sequence where X represents any amino acid) that binds two zinc atoms (Kwan, Gell et al. 2003). A typical topology of PHD and zinc coordination by the $Cys_4HisCys_3$ motif is shown in Fig. 2.2 for ING4. In general, PHD zinc fingers are believed to mediate protein-protein interactions that are critical for the function of proteins in which they are found (Aasland, Gibson et al. 1995).

These protein-protein interactions, in turn, may be regulated by a variety of mechanisms including posttranslational modifications and allosteric activation by small molecules. For example, in 2006, PHDs of various proteins including ING1 (Martin, Baetz et al. 2006; Taverna, Ilin et al. 2006), ING2 (Pena, Davrazou et al. 2006; Shi, Hong et al. 2006), and ING4 (Palacios, Garcia et al. 2006) were shown to recognize lysine trimethylation of histone 3. Additionally, the PHD of ING2 has been demonstrated to bind a subset of phosphoinositides (PtdInsPs) that are involved in cell signaling (Gozani, Karuman et al. 2003). Thus, the ING family proteins are able to regulate transcription by altering chromatin structure and, in turn, might be regulated by distinct stimuli. What governs the mechanism and the outcome of individual ING family member's effect on transcription, however, is protein specific.

While ING proteins are known to reside in different chromatin-modifying protein complexes, a given ING's specificity for a certain complex is determined by the primary amino acid sequence or splice variations of INGs (Feng, Hara et al. 2002). For example, N-terminal regions of ING proteins differ from one another though some sequence motifs are conserved (He, Helbing et al. 2005). These varying N-terminal regions are predicted to determine the specific protein-protein interactions and the function of the individual ING

protein. ING proteins, except ING1, also contain a Leucine Zipper-like (LZL) motif. Although the precise function is not clear, LZL motifs are predicted to mediate protein-protein interactions by generating a hydrophobic surface (He, Helbing et al. 2005). Even the splice variants of the same gene that differ only at the N-termini might have different, even opposing, functions as seen for ING1 in regulation of histone acetylation (Vieyra, Loewith et al. 2002) and apoptosis (Vieyra, Toyama et al. 2002). ING1 can also regulate apoptosis as its p32 isoform has a unique proliferating cell nuclear antigen (PCNA)-interacting protein (PIP) domain which is required for enhanced p32ING1-PCNA interaction upon exposure to UV-light and subsequent induction of apoptosis (Scott, Bonnefin et al. 2001).

ING family proteins have been implicated in a variety of different cellular processes including; cell growth and proliferation, senescence, cell cycle arrest, apoptosis, DNA repair, chromatin-remodeling, chromatin-dependent transcriptional activation, and repression (Gong, Suzuki et al. 2005; Shi and Gozani 2005; Russell, Berardi et al. 2006). Although ING proteins themselves do not possess any enzymatic activity, co-purification with histone acetyltransferase (HAT) or histone deacetyltransferase (HDAC) activities suggested that ING family proteins are components of HDAC and/or HAT complexes (Feng, Hara et al. 2002; Campos, Chin et al. 2004; Gong, Suzuki et al. 2005; Shi and Gozani 2005; Russell, Berardi et al. 2006). Unambiguous support for these models came from the biochemical purification of ING3 (Doyon, Selleck et al. 2004) and ING1 (Skowyra, Zeremski et al. 2001) as components of NuA4 HAT and mSin3A-HDAC1/2 complexes, respectively. Although there is little sequence similarity between N-terminal regions of ING proteins, they have recently been designated as potential chromatin regulatory (PCR) domains to reflect their potential

involvement in differential chromatin modifying enzyme (HATs and/or HDACs) binding (He, Helbing et al. 2005).

ING4, the fourth member of the ING family, was initially identified by its sequence similarity to ING1 (Shiseki, Nagashima et al. 2003). In agreement with the general function of ING family proteins, expression of exogenous ING4 reduced colony-forming efficiency, inhibited cell growth, and induced cell death in a p53-dependent manner of transfected cells (Shiseki, Nagashima et al. 2003), and in another study shown to induce cell cycle arrest at G2/M transition (Zhang, Xu et al. 2004). ING4, like the rest of the ING proteins, has received attention as a candidate tumor suppressor protein and has recently been associated with regulation of brain tumor growth and angiogenesis (Garkavtsev, Kozin et al. 2004). In tissue samples isolated from human glioblastoma tumors, an inverse relationship between ING4 mRNA level and tumor aggressiveness was observed. Gene expression analysis of U87MG glioblastoma cells using an angiogenesis-related gene microarray revealed that pro-angiogenic targets of NF-κB –IL6, IL8, and COX2– were downregulated when ING4 was stably overexpressed and upregulated when ING4 was repressed by stably transfected antisense-ING4 expressing construct (Garkavtsev, Kozin et al. 2004). ING4 was also identified in a screen looking for genes that can revert loss of contact inhibition caused by MYCN proto-oncogene (Kim, Chin et al. 2004). Consistent with being a tumor suppressor gene, mutations and reduced expression of ING4 have been observed in tumors and cancer cell lines (Garkavtsev, Kozin et al. 2004; Kim, Chin et al. 2004; Gunduz, Nagatsuka et al. 2005). Since its identification in 2003, ING4 has been implicated in regulation of cell proliferation and death (Shiseki, Nagashima et al. 2003), tumor growth and angiogenesis

(Garkavtsev, Kozin et al. 2004), contact inhibition (Kim, Chin et al. 2004), and cell cycle arrest (Zhang, Xu et al. 2004). Our laboratory became interested in ING4 since we observed that ING4 is a HIF Proyl-Hydroxylase-2 (HPH-2) interacting protein (Chapter 1) and further characterized its role in regulation of cellular hypoxic response pathway (Ozer, Wu et al. 2005).

Experimental Procedures

Recombinant Protein Expression and Purification

Protein coding sequences for ING1 isoforms p24 (GenBank accession #: NM_198218), p27 (NM_198217), and p32 (NM_198219), ING2 (NM_001564), ING3 (NM_019071), ING4 (NM_016162), ING5 (NM_032329), HPH-2 (AF229245), and FIH-1 (NM_053046) were amplified by PCR and cloned into appropriate restriction sites of bacterial and mammalian expression plasmids. pHIS-, pGST-, pMBP-, and pGβ1-parallel (Sheffield, Garrard et al. 1999; Amezcua, Harper et al. 2002), and pET28A (Novagen) plasmids were used for bacterial expression of fusion proteins. For mammalian expression of C-terminal V5- or un-tagged proteins pcDNA3.1/V5-HisA (Invitrogen) and for N-terminal 3XFLAG-tagged proteins p3XFLAG-CMV-10 (Sigma-Aldrich) plasmids were used. For yeast two-hybrid experiments pVJL10 and pGAD-NotII vectors were used. Every construct has been confirmed by DNA sequencing.

Recombinant proteins expressions were induced in *E.coli* BL21(DE3)RIL strain (Stratagene) with addition of IPTG to final concentration of 200 μM in LB media containing

antibiotics (100 μg/ml ampicillin or 50 μg/ml kanamycin). Bacterial cultures were grown for 4-16 hours post-induction at 20-25 °C and bacterial pellets were frozen at -80 °C.

Bacteria expressing Gβ1-ING4 fusion protein were lysed in buffer containing 20 mM Tris.Cl pH 8.0, 50 mM NaCl, 5 mM β-mercaptoethanol, 100 μM $ZnCl_2$, 1 mg/ml Lysozyme, and 1:200 Protease Inhibitor Cocktail (Sigma) and purified over Source Q resin (Amersham Pharmacia) with the same buffer. The Gβ1 tag was removed by TEV protease digestion followed by ion exchange chromatography using Mono S resin (Amersham Pharmacia) equilibrated with 50 mM $NaPO_4$ pH 5.9, 5 mM β-mercaptoethanol, and 10 μM $ZnCl_2$. ING4 containing fractions were combined (300 mM NaCl) and stored at 4 °C. Recombinant FIH-1 and catalytic domain of HPH-2, amino acids 181–426 (HPH-2C), were expressed with N-terminal 6XHIS-tag using pHIS parallel vector. Bacterial lysates (50 mM Tris.Cl pH 8.0, 300 mM NaCl, 5 mM β-mercaptoethanol, 1mg/ml lysozyme, 5 μg/ml DNase I) were incubated with Ni-NTA resin and eluted with lysis buffer containing 250 mM imidazole. Recombinant proteins were then subjected to anion exchange chromatography using a Mono Q column (Amersham Pharmacia) with 20 mM Tris.Cl pH 8.0, 5 mM β-mercaptoethanol buffer and eluted with NaCl gradient. TEV digestion, Ni-NTA and size exclusion chromatography (Superdex 75 column (Amersham Pharmacia)) with 20 mM Tris.Cl pH 8.0, 50 mM NaCl, 5 mM β-mercaptoethanol buffer were used to eliminate 6XHIS-tag and un-cut protein. Recombinant HIF-1α ODD was expressed N-terminally tagged with 6XHIS and purified similar to FIH-1 and HPH-2C using Ni-NTA resin, Mono Q, and Superdex 75 columns with following buffers; lysis buffer: 20 mM Tris.Cl pH 7.5, 100 mM NaCl, 5 mM β-

mercaptoethanol, 0.5 mM PMSF, 0.2% NP-40, 1mg/ml Lysozyme, Mono Q column buffer: 20 mM Tris.Cl pH 7.5, 5 mM β-mercaptoethanol eluted with NaCl gradient, and size exclusion column buffer: 20 mM Tris.Cl pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol. Final fractions containing untagged recombinant proteins were combined and used directly or stored at -80 °C after addition of glycerol to final concentration of 20%.

For size exclusion chromatography of purified recombinant proteins, 100 μg of each protein was resolved on Superdex 200 (Amersham) column with buffer containing 20 mM Tris.Cl pH 7.5, 100 mM NaCl, 5 mM β-mercaptoethanol. For combinations of proteins, protein mixtures were incubated 10 min on ice prior to size exclusion chromatography.

Yeast Two-Hybrid Assay

The yeast strain L40 (*MATa, trp1, leu2, his3, LYS2::lexA-HIS3, URA3::lexA-LacZ*) was used to test protein-protein interactions in yeast. L40 cells normally grown on YPD media were transformed with vectors encoding HPH-2C fused to the LexA DNA-binding domain (DBD) in pVJL10 vector and ING4 fragments or other ING family proteins fused to the GAL4 activation domain in pGAD-NotII vector. Doubly transformed cells were selected on plates with synthetic minimal medium (2% glucose, 0.67% nitrogen base without amino acids (Difco) supplemented with SDA Medium–LEU–TRP (Q-BIOgene)). Positive protein–protein interactions were assayed by transactivation of LacZ and HIS reporter genes in two independent double-transformants. LacZ β-galactosidase activity were assayed with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in a filter-lift assay, whereas HIS expression was judged by growth on synthetic medium lacking –LEU–TRP–HIS.

Cell Culture: Transient Transfections and Luciferase Assay

HeLa cell lines were maintained in Dulbecco's modified Eagle's medium (HyQ DME, HyClone) containing high glucose supplemented with 10% FBS (Gemini Biological Products) in the presence of 5% CO_2 at 37 °C. Cells were maintained under hypoxic conditions at 37 °C within a humidified hypoxic chamber (Coy Laboratory Products) filled with 1% O_2, 5% CO_2, and 94% N_2 air for 15 h unless indicated otherwise. Transient overexpression were achieved by transfection of ING4 in pcDNA3.1-V5/HisA or p3XFLAG-CMV-10 vectors to HeLa cells or HeLa derived reporter cell lines with Lipofectamine and Plus reagent (Invitrogen) according to manufacturer's protocol. In 3XHRE-tk-Luciferase reporter construct, expression of luciferase is controlled by 3 tandem repeats of Hypoxia Response Element (HRE) derived from EPO gene enhancer.

Antibodies and Western Blot Analysis

Polyclonal antiserum was obtained from two rabbits per protein immunized with HPH-2C, ING4, FIH-1, or HIF-β (a. acids 1-140) recombinant proteins. Endogenous HPH-1 or HPH-3 proteins were not detected by HPH-2 antiserum, whereas upon overexpression a weak cross-reactivity with ING1 and ING5 was observed for ING4 antiserum. HIF-β antiserum failed to detect HIF-β protein in Western Blot, however, it did effectively immunoprecipitated HIF-1α in co-immunoprecipitation (Co-IP) and HRE fragments in chromatin immunoprecipitation (ChIP) experiments (see below). Polyclonal antiserum against HPH-3 (Novus Biologicals), mouse monoclonal antibodies to p-ATF-2, annexin I

(Santa Cruz Biotechnology), and HIF-1α (BD Transduction Laboratories) were purchased. Cells were lysed with SDS sample buffer, resolved by SDS PAGE and transferred to Hybond C-extra membrane for Western Blot analysis. Primary antibodies were detected by species-specific HRP-conjugated secondary antibodies (Jackson ImmunoResearch) with enhanced chemiluminescence (Amersham Biosciences).

Co-immunoprecipitation

HeLa cells transfected with ING4 in the p3XFLAG-CMV10 vector (Sigma) were lysed with 20 mM Tris.Cl pH 7.5, 100 mM NaCl, 5 mM 2-mercaptoethanol, 1% Nonidet P-40, and Protease Inhibitor Cocktail (Sigma) 24 h post-transfection. Lysates were precleared with Protein A-agarose beads (Roche Diagnostics) and incubated in the presence of preimmune serum or HPH-2C immune serum overnight at 4°C. Antibody–protein complexes were precipitated with Protein A-agarose beads, washed extensively with lysis buffer, and eluted with SDS sample buffer. Co-immunoprecipitated 3XFLAG-ING4 was detected by Western blotting using α-FLAG M2 antibody (Sigma).

GST Pull-Down

[^{35}S]-labeled proteins were prepared in the TNT Coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S]-Methionine (Amersham Biosciences). Lysates containing [^{35}S]-labeled proteins were incubated with GST fusion proteins immobilized on glutathione sepharose 4B resin (Amersham Pharmacia) for 1 h at 4°C. After several washes with buffer containing 20 mM Tris.Cl pH 7.5, 200 mM NaCl, 1 mM DTT, 0.5% NP-40, bound proteins

were eluted by boiling in SDS sample buffer, resolved by SDS PAGE, and exposed to PhosphorImager screen. PhosphorImager screens were scanned by Typhoon PhosphorImager and quantitated by ImageQuant software (Molecular Dynamics).

Subcellular Fractionation

HeLa cells were washed with ice-cold PBS and harvested by scraping. Cell pellets were resuspended in 10mM HEPES-KOH pH 7.5, incubated on ice for 10 min, and centrifuged at 2,400xg at 4°C for 5 min. Pellets were washed with 25 mM HEPES-KOH pH 7.5, 3 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, and 1:200 Protease Inhibitor Cocktail and resuspended in one packed cell volume (PCV) of the same buffer. Cells were lysed by passage through a 25-gauge needle and centrifuged for 30 sec. The cytosolic supernatant was removed, and the pellets containing nucleus were washed three times before resuspension in 2 nuclear pellet volume of 20 mM HEPES-KOH pH 7.5, 400 mM KCl, 1 mM DTT, and 10% glycerol and incubated at 4°C for 30 min with gentle stirring. The samples were centrifuged at 200,000xg for 45 min at 4°C to separate the extracted nuclear proteins from insoluble material.

HIF Prolyl Hydroxylase Activity Assay: [^{14}C]-2-Oxoglutarate Decarboxylation

Hydroxylation of candidate HPH substrate proteins was measured by a modified [^{14}C]-2-oxoglutarate decarboxylation assay derived from (Kivirikko and Myllyla 1982). Briefly, 1-ml reaction containing 3.0 μM peptide substrate (HIF-1α a.acids 556–574) or recombinant ING4 protein was incubated with recombinant HPH-2C enzyme in the presence

of 50 mM Tris.Cl pH 8.0, 2 mg/ml BSA, 0.2 mg/ml catalase, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 64 μM [^{14}C]-2-oxoglutarate [14.6 nCi/nmol specific activity] (Perkin Elmer), 50 μM ascorbate, and 20 μM $FeSO_4$ in a sealed 15-ml tube. After one hour incubation at room temperature with gentle shaking, the pH of the solution was lowered to ~2 with diluted $HClO_4$. Released [^{14}C]-CO_2 was captured by 3M Whatman paper saturated with 10 M NaOH and measured by a scintillation counter (Beckman).

HIF Prolyl Hydroxylase Activity Assay: [^{35}S]-pVHL Pull-down

Biotinylated HIF-1α (residues 556-574) peptide substrate bound to Neutravidin beads (Pierce) was incubated with recombinant HPH-2C enzyme at 30 °C for 30 min in Reaction Buffer (20 mM Tris.Cl pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 2 mM ascorbate, 2 mM 2-oxoglutarate, 1 mM DTT, 0.1 mM $FeSO_4$). Beads were washed three times with NETN Buffer (20 mM Tris.Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) before and after incubation with [^{35}S]-labeled pVHL protein in EBC Buffer (50 mM Tris.Cl pH 8.0, 120 mM NaCl, 0.5% NP-40). Washed beads were resuspended in scintillation fluid and radioactivity was measured by scintillation counting.

RNA Interference

HeLa cells were plated onto 24-well plates ($3x10^4$ cells per well) 16 h before transfection, and 200 nM small interfering RNA (siRNA) duplexes (Dharmacon) were transfected with Oligofectamine reagent (Invitrogen) according to manufacturer's protocol. Growth media was replaced 16–20 h post-transfection. Cells were maintained either under

normoxic conditions (20% O_2) for 72 h or under normoxic conditions for 57 h followed by 15 h of hypoxia (1% O_2). Total RNA was extracted with STAT-60 reagent (Tel-Test Inc.) according to manufacturer's protocol, resuspended in 0.1 mM EDTA solution, and quantitated by UV spectrometer for Northern Blot and quantitative real-time PCR (qRT-PCR) analyses. For HIF activity measurements, HeLa cells either stably or transient transfected with 3XHRE-tk-Luciferase reporter construct were used. Transient transfection of reporter construct was done 51 h after siRNA transfection using Lipofectamine and Plus reagents (Invitrogen). Luciferase assays were performed as described in (Bruick 2000). Briefly, cells were lysed with 30 mM Tricine pH 7.8, 8 mM Mg.Acetate, 0.2 mM EDTA, 100 mM β-mercaptoethanol, 1% Triton X-100 buffer. Lysates were mixed with equal volume of lysis buffer containing 3 mM ATP, 1 mM Coenzyme A, and 1 mM Luciferin and emitted luminescence was measured in opaque 96-well plates with a luminescence microplate reader (Biotek). siRNA duplexes were composed of the following oligonucleotides: ING4 #1, UGAGGGACCUAGACCAAAGdTdT and CUUUGGUCUAGGUCCCUCAdTdT; ING4 #2, GAACGGAAGAAGAAAUAGAdTdT and UCUAUUUCUUCUUCCGUUCdTdT; GFP, GGCUACGUCCAGGAGCGCACC and UGCGCUCCUGGACGUAGCCUU.

Northern Blot

10 μg of total RNA from each sample was used for detection of NIP3 and AK3 mRNA and 3 μg for detection of actin mRNA and EtBr staining for ribosomal RNA. RNA samples loaded with RNA Loading buffer (50% formaldehyde, 20% formamide, 20% dye buffer (50% glycerol, 10 mM EDTA pH 8.0, 0.25 bromophenol blue, 0.25% xyxlene cyanol),

and 10% 10X MOPS buffer(0.4 M MOPS-NaOH pH 7.0, 0.1 M Na.Acetate, 0.01 M EDTA)) at 1:5 ratio were resolved on 1.2% agarose-formaldehyde gel (1.2% agarose, 1.8% formaldehyde, in 1X MOPS buffer) using 1X MOPS buffer. After electrophoresis, RNA gel was washed 4 times 5 min with ddH_2O and once for 5 min with 20X SSC buffer (3.0 M NaCl, 0.3 M sodium citrate pH 7.0). Resolved RNA was transferred to Nytran Supercharge nylon membrane (Schleicher & Schuell Bioscience) pre-equilibrated with ddH_2O and 20X SSC 5 min each by diffusion overnight using 20X SSC buffer. Transferred RNA was UV cross-linked to membrane, pre-hybridized with Rapid-Hyb Buffer (Amersham) at 62 °C for 1-2 h. Protein encoding fragments of NIP3, AK3, and actin genes, around ~600 bp in size, were radiolabeled with Rediprime II random prime labeling system (Amersham) supplemented with Redivue γ-[32P]-ATP (GE Healthcare). Labeled-probes purified using ProbeQuant G50 microspin columns (Amersham) were added directly into pre-hybridization buffer and hybridized overnight at 62 °C. Membranes were washed twice with 70 °C-equilibrated 0.2X SSC, 0.1% SDS buffer and once with 70 °C-equilibrated 0.1X SSC, 0.1% SDS buffer at 62 °C for 20 min each. Membranes were then exposed to PhosphorImager screens.

Quantitative Real-Time PCR (qRT-PCR)

Contaminating DNA in RNA samples was eliminated by DNase I treatment prior to reverse-transcription reaction. 2 µg total RNA was incubated 30 min at 37 °C and 10 min at 75 °C with 6.4 units of RNase-free DNase I (Roche) in the presence of 4.2 mM $MgCl_2$ in 40 µl total volume. 1µg of DNase I-treated RNA was reverse-transcribed with SuperScript II

reverse transcriptase (Invitrogen) using random hexamers (Roche) according to manufacturer's protocol. Resulting cDNA was diluted 10-fold with ddH_2O to 2 ng/µl and 10 µl of this was mixed with 30 µl of real-time PCR mix (20 µl 2X SYBR Green PCR Master Mix (Applied Biosystems), 4.8 µl 1.25 µM gene specific primer mix, 5.2 µl ddH_2O). 10 µl of this final mix was aliquoted in triplicate into wells of 384-well plate, data acquisition for PCR reactions in real-time were done with 7900HT Fast Real-Time PCR System, and analyzed with SDS 2.2.2 software (Applied Biosystems). mRNA levels relative to cyclophilin mRNA in each sample were calculated using ΔΔCt method described in (Bookout and Mangelsdorf 2003). The following gene-specific primer pairs were designed using Primer Express software (Applied Biosystems): VEGF, 5'-GAGGGCAGAATCATCACGAA-3'and 5'-GGCAGTAGCTGCGCTGATAG-3'; PGK1, 5'-TTGGCACTGCTCACAGAGC-3' and 5'-GCCTTCTGTGGCAGATTGAC-3'; LDHA, 5'-CACAAGCAGGTGGTTGAGAGT-3' and 5'-CCCAGGATGTGTAGCCTTTG-3'; GLUT1, 5'-CGGGTTGTGCCATACTCATG-3' and 5'-ACATCCAGGGTAGCTGCTCC-3'; cyclophilin, 5'-TGCCATCGCCAAGGAGTAG-3' and 5'-TGCACAGACGGTCACTCAAA-3'.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation protocol was adapted from (Chakrabarti, James et al. 2002). HeLa cells or HeLa cell line stably transfected with the 3XHRE-tk-Luciferase reporter construct were maintained under hypoxic conditions (1% O_2) for 15 h. Crosslinking of proteins to DNA was achieved by addition of formaldehyde to culture media to 1% final

concentration in 15 min at room-temperature and terminated by 5 min wash with 0.125 M glycine in 1X PBS. Cells were lysed in 3.5 packed cell volume of lysis buffer (50 mM Tris.Cl pH 8.1, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 5 mM EDTA) 20 min on ice. Lysate sonicated to fragment DNA into 200-1000 bp pieces was centrifuged at 12,800 rpm for 10 min at 4 °C to remove cell debris. Supernatants pre-cleared with Salmon Sperm DNA/Protein A-agarose mixture were incubated for 20 h at 4 °C with indicated antibodies at 1/200 dilution or without any antibody. Antibody-chromatin complexes precipitated with Salmon Sperm DNA/Protein A-agarose mixture were then washed once with Low Salt (20 mM Tris.Cl pH 8.1, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA), High Salt (20 mM Tris.Cl pH 8.1, 1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA), LiCl buffers (10 mM Tris.Cl pH 8.1, 1% NP-40, 1% deoxycholate, 250 mM LiCl, 1 mM EDTA), and twice with TE buffer (10 mM Tris.Cl pH 8.0, 1 mM EDTA). Cross-linked protein-DNA complexes were eluted twice with 250 µl of fresh elution buffer (1% SDS, 0.1 M $NaHCO_3$). 20 µl of 5 M NaCl solution was added to combined eluates and crosslinking was reversed by incubation at 65 °C for 24 h. DNA fragments separated from proteins by Proteinase K digestion, Phenol/Chloroform extraction, and EtOH precipitation were dissolved in TE buffer and used for PCR amplification of target promoter regions. 227, 305, and 219 bp promoter regions were amplified with the following primers sets: 3XHRE, 5'-GTGCAGGTGCCAGAACATT-3' and 5'-CGGTAGGTCGAGAGGTCAGA-3'; COX2 (Nie, Pang et al. 2003), 5'-AAGACATCTGGCGGAAACC-3' and 5'-ACAATTGGTCGCTAACCGAG-3'; and IL6, 5'-ACGACCTAAGCTGCACTT-3' and 5-'GCAGAATGAGCCTCAGAC-3'. PCR products were separated by electrophoresis on a

1.5% agarose gel and stained with ethidium bromide. Relative band intensities were quantitated with QUANTITY ONE software (Bio-Rad).

Results

HPH-2 interacts with ING4

ING4 was originally identified as an HPH-2 interacting protein in a yeast two-hybrid screen performed by Richard Bruick and Leeju Wu using the catalytic C-terminal domain of HPH-2 (HPH-2C) as bait (Chapter 1). Since the ING protein family contains 4 additional members (excluding alternatively spliced isoforms), there existed the possibility of interactions between HPH-2 and INGs other than ING4. To address this possibility, we cloned ING1 (isoforms p24, p27, and p32) and ING2 into pGAD-NotII vector to be expressed as GAL4 activation domain (GAL4-AD) fusions. Positive protein-protein interactions were tested in yeast L40 strain doubly transformed with GAL4 AD-ING family protein and LexA DNA binding domain-HPH-2C (LexA DBD-HPH-2C) expressing constructs by X-gal staining for LacZ reporter expression and by growth on SD –Trp –Leu –His media for His reporter gene expression (Fig. 2.3A). HPH-2C did not induce expression of either the LacZ or His reporter gene by itself; however, both reporters were induced as indicated by blue color in X-gal staining and growth on SD –Trp –Leu –His media when co-expressed with ING4. On the other hand, ING1 isoforms and ING2 proteins were unable to induce expression of reporters when co-expressed with HPH-2C indicating that ING4 is the only ING capable of a selective interaction with the catalytic domain of HPH-2 (HPH-2C) in yeast.

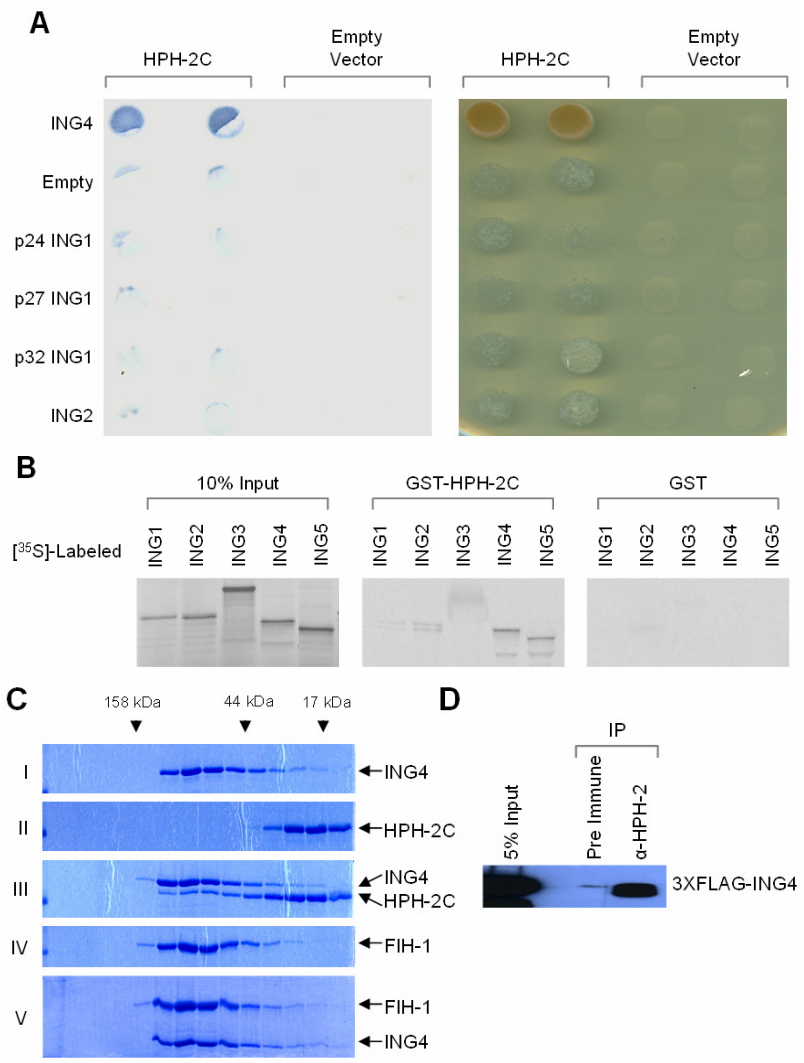


Figure 2.3 ING4 interacts with HPH-2.
(A) L40 yeast strain was transformed with HPH-2C and ING family protein (ING4, ING1 isoforms p24, p27, and p32, and ING2) expressing vectors. Protein-protein interactions were assayed by X-gal staining for LacZ reporter expression (left panel) and growth on –Leu –Trp –His selective media. Two independent clones for each combination were assayed and corresponding empty vectors transformations served as negative controls.
(B) Interaction between HPH-2C and ING family proteins (ING1-5) were assayed by GST pull-down experiment where radiolabeled ING proteins were pulled down with GST-HPH-2C (middle panel) or GST (right panel) proteins. 10% of each radiolabeled ING protein used for the assay was shown on the left panel.
(C) Purified recombinant ING4, HPH-2C, and FIH-1 proteins were separated on Superdex 200 size exclusion column individually or combined. A positive protein-protein interaction lead to a shift of proteins to higher molecular weight fractions.
(D) 3XFLAG-ING4 protein transiently expressed in HeLa cells was co-immunoprecipitated together with endogenous HPH-2 protein using α-HPH-2 antibody but not with pre immune serum. 5% of the lysate used for co-immunoprecipitation experiment was shown. Figures 3, 4, 5, 6, 7, 9, and 11 and corresponding figure legends were taken from (Ozer, Wu et al. 2005).

Interactions between HPH-2 and ING family proteins was further tested in GST pull-down experiments where [^{35}S]-labeled ING family proteins (ING1-5) were pulled-down with immobilized GST-HPH-2C recombinant protein. ING4, ING5 and to a lesser extent ING1, ING2, and ING3 were pulled-down by GST-HPH-2C but not by GST (Fig. 2.3B). Comparison with the inputs for each protein suggests that ING4 has higher affinity towards HPH-2C than any other ING family proteins *in vitro*. A direct protein-protein interaction between ING4 and HPH-2C proteins was also demonstrated by the use of recombinant proteins and size exclusion chromatography (Fig. 2.3C). Purified recombinant ING4 and HPH-2C proteins were resolved on Superdex 200 size exclusion column either separately or in combination. ING4, a 29 kDa protein, eluted with a peak in ~90 kDa molecular weight fraction –estimated by the elution profile of protein size marker– consistent with a homotrimer. ING4, although still larger than its calculated size, was also present in smaller molecular weight fractions. HPH-2C, a 25 kDa protein, eluted at predicted molecular weight fractions as a monomer. However, when combined with ING4, HPH-2C eluted in higher molecular weight fractions that contained ING4 and at the same time it shifted the ING4 peak to ~120 kDa consistent with the addition of a 25 kDa protein to ING4 trimer. To eliminate the possibility that the interaction between HPH-2 and ING4 was the result of a non-specific protein aggregation, purified recombinant FIH-1, HIF asparaginyl hydroxylase that is closely related to HIF prolyl hydroxylases, was used as a control. As demonstrated by others (Dann, Bruick et al. 2002; Lancaster, McNeill et al. 2004), FIH-1 (42 kDa protein) eluted as a dimer by itself and neither ING4 nor FIH-1 affected the elution profile of one another. In addition to *in vitro* and yeast two-hybrid experiments, *in vivo* HPH-2 and ING4

interaction was confirmed by co-immunoprecipitation experiments. Transiently expressed N-terminal 3XFLAG-tagged ING4 (3XFLAG-ING4) protein was co-immunoprecipitated with endogenous HPH-2 protein using HPH-2 immune serum but not with pre-immune serum (Fig. 2.3D).

HPH-2 interacts with ING4 Plant Homeodomain (PHD)

ING family proteins share a highly conserved C-terminal Plant Homeodomain (PHD) and differ immensely at the N-terminal region. We wanted to identify the region of ING4 that associates with HPH-2C and to this end N- and C-terminal deletions of ING4 were made. N-terminal deletions as far as the PHD domain (ING4 191-249) retained positive interaction with HPH-2C as assayed in yeast by X-gal staining and growth on SD –Leu –Trp –His media (Fig. 2.4A). However, the N-terminal fragment of ING4 lacking PHD (ING4 1-198) lost the ability to interact with HPH-2C. Mutation of a conserved cysteine residue to serine (C226S) designed to disrupt the PHD fold (Fig. 2.2), abrogated the HPH-2C interaction indicating the importance of properly folded PHD zinc finger. In *in vitro* GST pull-down experiments, deletion of PHD in ING4 significantly attenuated pull-down by GST-HPH-2C compared to full-length ING4 (Fig. 2.4B). Neither full-length nor PHD-deleted ING4 was pulled-down by GST. Taken together, these data support the hypothesis that the PHD of ING4 is required and sufficient for HPH-2 interaction.

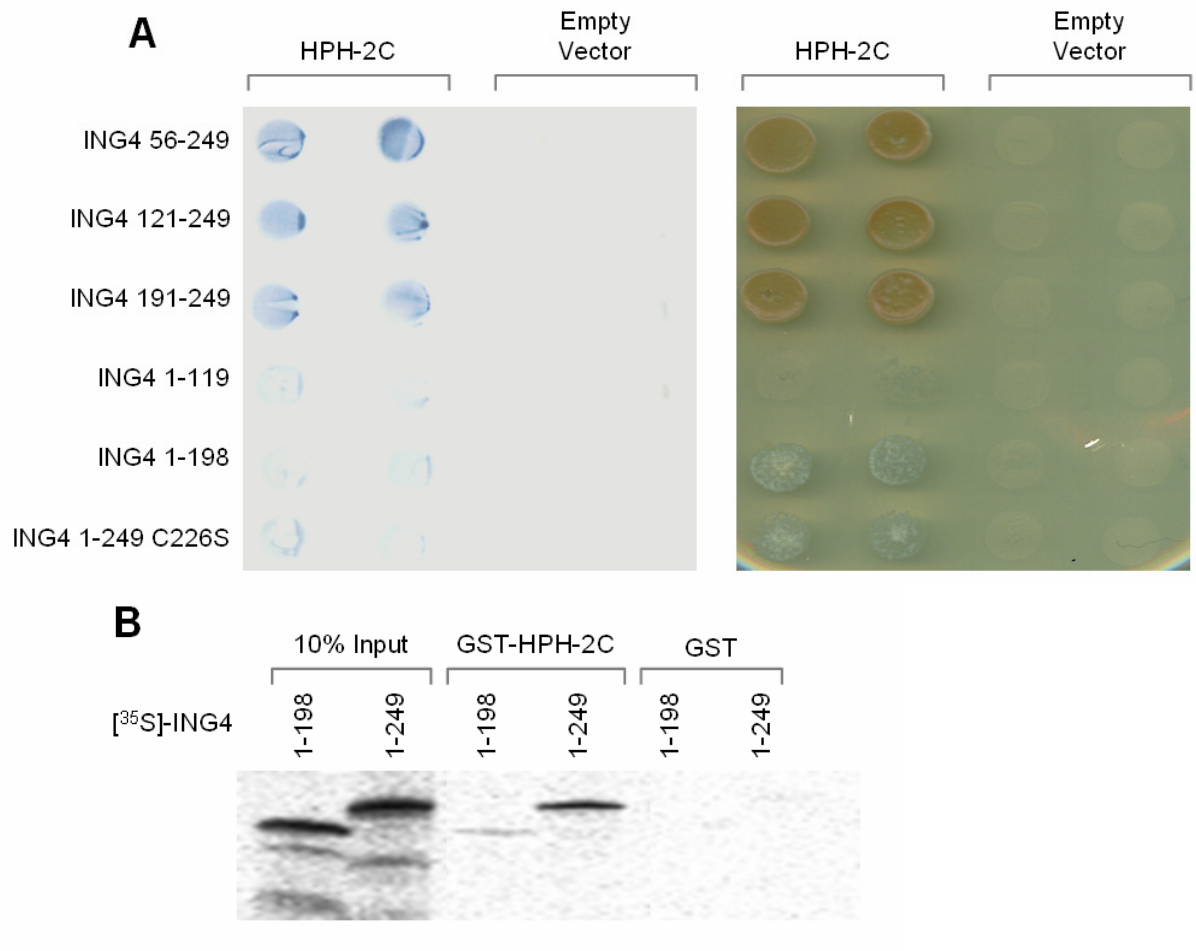


Figure 2.4 HPH-2C interacts with ING4 Plant Homeodomain (PHD).
(A) L40 yeast strain transformed with HPH-2C and mutant ING4 protein expression vectors. Protein-protein interactions were assayed by X-gal staining for LacZ reporter expression (left panel) and growth on –His–Trp–Leu selective media. Two independent clones for each combination were assayed and corresponding empty vector transformations served as negative controls.
(B) Interaction between HPH-2C and full-length (1-249) or PHD deleted (1-198) ING4 proteins were assayed by GST pull-down experiment where radiolabeled ING4 proteins were pulled down with GST-HPH-2C or GST proteins bound to glutathione resin. 10% of each radiolabeled ING4 protein fragment used for the assay was shown on left panel.

Both HPH-2 and ING4 are present among HeLa nuclear proteins

Though a positive interaction has been observed between transiently expressed ING4 and endogenous HPH-2 proteins, localization of ING4 and HPH-2 into separate subcellular compartments may prevent the two endogenous proteins from forming a complex *in vivo* (Fig. 2.3A). ING4 (Zhang, Wang et al. 2005) and other ING family proteins (Feng, Hara et al. 2002) are localized to nucleus by virtue of their strong nuclear localization signal (Fig. 2.1), whereas the HPH-2 protein has been reported to be localized exclusively in cytoplasm (Metzen, Berchner-Pfannschmidt et al. 2003). To address this problem, HeLa cells exposed to normoxic and hypoxic conditions were fractionated into crude cytoplasmic and nuclear fractions and proteins in each fraction were analyzed by western blot using ING4 and HPH-2 immune serum (Fig. 2.5). ING4 was present exclusively in nuclear extracts and hypoxic exposure had no effect on it localization or protein level. Contrary to earlier published results (Metzen, Berchner-Pfannschmidt et al. 2003), HPH-2 is present among both nuclear and cytoplasmic proteins (Fig. 2.5) allowing endogenous ING4 and HPH-2 proteins to form a nuclear complex *in vivo*. Consistent with being a HIF target gene (Metzen, Stiehl et al. 2005), the HPH-2 protein level was also increased under hypoxic conditions. In the same extracts we also analyzed distribution of HPH-3 and FIH-1 proteins, in agreement with published results (Metzen, Berchner-Pfannschmidt et al. 2003; Stiehl, Wirthner et al. 2006), FIH-1 protein was detected mostly in cytoplasmic extract and neither its level nor its localization was affected by hypoxia treatment, while HPH-3 was detected mainly in nuclear extract and its protein level was induced by hypoxia treatment. Specific antibodies against the

phosphorylated-ATF-2 transcription factor and annexin I were used to asses the integrity of nuclear and cytoplasmic fractions, respectively.

ING4 is not an HPH-2 substrate

Identification of known HPH-2 substrates –HIF-α fragments encompassing the Oxygen-dependent Degradation Domain (ODD)– in addition to ING4 raised the possibility that ING4 might be hydroxylated by HPH-2. Although the ING4 protein sequence lacks the LXXLAP hydroxylation motif found in HIF-α subunits (Masson, Willam et al. 2001), tolerance of HPH enzymes to mutations within this motif for hydroxylation (Huang, Zhao et al. 2002) led us to question whether ING4 can be hydroxylated by HPH-2. To this end, we adopted an assay previously used to measure activity of collagen-modifying Prolyl 4-Hydroxylases (P4Hs) (Kivirikko and Myllyla 1982), which are Fe(II)- and 2-oxoglutarate-dependent dioxygenase much like HPH enzymes. During the hydroxylation of the primary substrate whether it be collagen or the HIF-α subunit, the 2-oxoglutarate co-substrate becomes hydroxylated and subsequently gets decarboxylated releasing CO_2 and succinnate as byproducts. By using [^{14}C]-labeled 2-oxoglutarate, we were able to measure hydroxylase activity via the evolution of radiolabeled CO_2. Incubation of HPH-2C with a peptide substrate derived from HIF-1α (residues 556-574) induced [^{14}C]-CO_2 release compared to its absence (Fig. 2.6). Unlike HIF-1α peptide substrates, incubation of recombinant ING4 protein with HPH-2C did not result in significant [^{14}C]-CO_2 release compared to reactions containing HPH-2C alone indicating HPH-2 is unable to hydroxylate ING4 *in vitro*. Furthermore, MALDI-TOF mass spectrometric analysis of ING4 protein fragments (residues

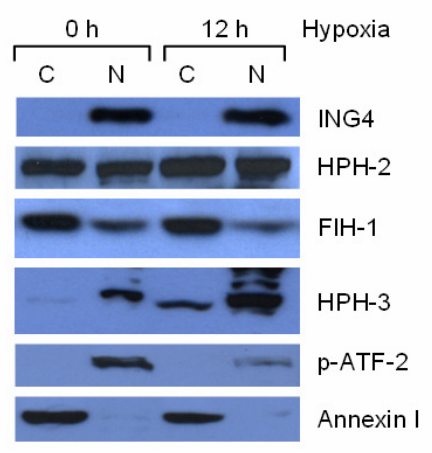
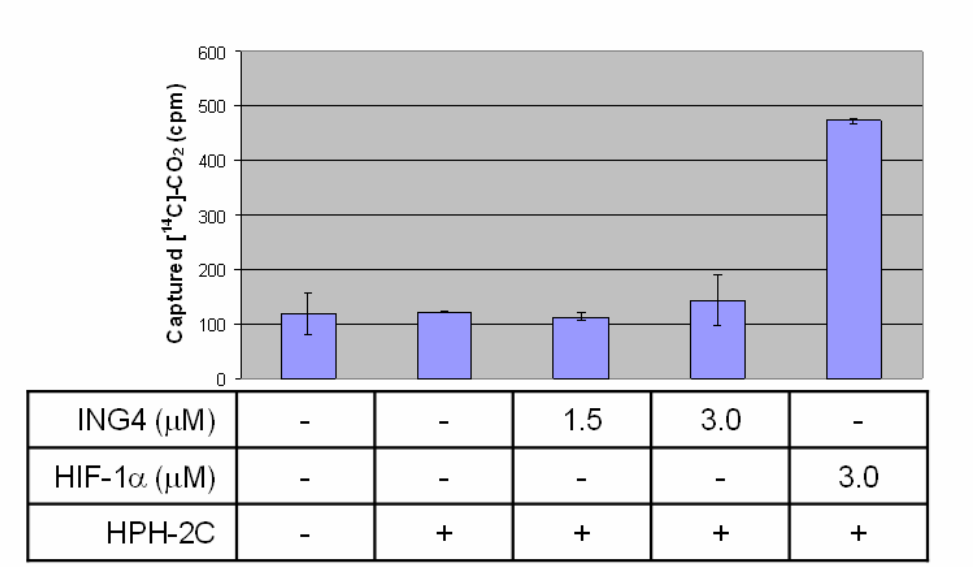


Figure 2.5 ING4 and HPH-2 are among the salt-extracted nuclear proteins.
HeLa cells grown under normoxic or hypoxic conditions for 12 h were fractionated into cytoplasmic and 0.4 M KCl nuclear protein extracts. Proteins were detected by western blot analysis using specific antibodies. Successful fractionation of nuclear and cytoplasmic extracts was assessed by detection of nuclear (phosphorylated-ATF-2) and cytoplasmic proteins (Annexin I).



ING4 (μM)	-	-	1.5	3.0	-
HIF-1α (μM)	-	-	-	-	3.0
HPH-2C	-	+	+	+	+

Figure 2.6 ING4 is hydroxylated by HPH-2C *in vitro*.
Hydroxylation of substrate by HPH-2C recombinant enzyme was assessed by the amount of [^{14}C]-CO_2 generated due to hydroxylation coupled decarboxylation of [^{14}C]-2-oxoglutarate co-substrate. Incubation of recombinant ING4 protein with HPH-2C enzyme did not increase generation of [^{14}C]-CO_2 as HIF-1α peptide substrate did at the same concentration.

121-249 and 191-249) incubated with HPH-2C under conditions suitable for hydroxylation did not reveal a mass increase of 16 Da expected due to hydroxylation (data not shown). Thus, these *in vitro* results do not support ING4 being a substrate for HPH-2 hydroxylation.

ING4 does not affect HPH-2 activity or HIF stability

Since ING4 did not serve as a HPH-2 substrate, we speculated that it might affect HPH-2 hydroxylase activity. To test this hypothesis, we used [^{35}S]-pVHL pull-down assays to measure the hydroxylase activity of HPH-2 in the presence and absence of ING4 protein. The [^{35}S]-pVHL pull-down assay relies on a hydroxylation-dependent interaction between radiolabeled pVHL protein and immobilized HIF-1α peptide substrate, and hydroxylase activity can thus be measured by the extent of captured pVHL protein. Control experiments demonstrated that addition of recombinant HPH-2 significantly increased pVHL protein capture when compared to reactions lacking HPH-2 enzyme (Fig. 2.7). When recombinant ING4 protein was added to the reactions at ratios of 0.1, 1, and 10 with respect to HPH-2C protein, there was no increase or decrease in the amount of captured [^{35}S]-pVHL. Therefore, we concluded that ING4 has neither allosteric nor substrate competitive effects on hydroxylase activity of HPH-2.

To test HPH-2 hydroxylase activity as a result of ING4 levels *in vivo*, transient overexpression of ING4 protein was performed in reporter cell lines (Fig. 2.8A). HeLa cells stably transfected with luciferase reporters were transiently transfected with ING4 expression construct and the luciferase activity was monitored under both normoxic and hypoxic conditions. Luciferase expression from 3XHRE-tk-Luciferase construct is dependent on

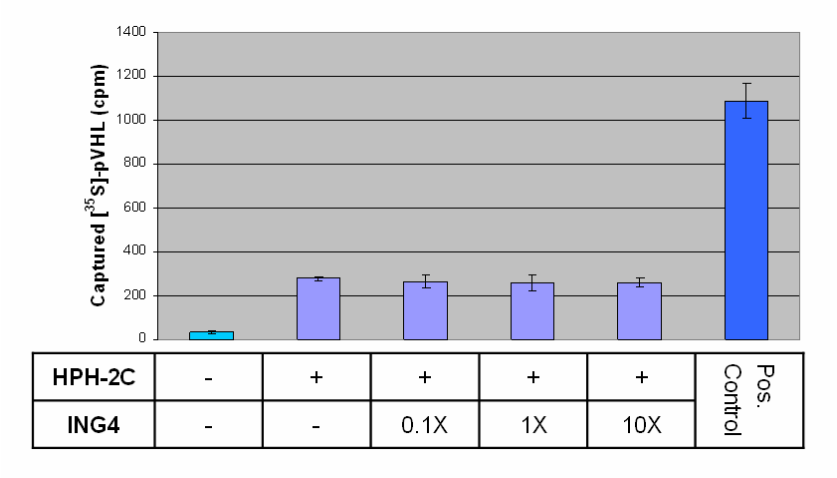


Figure 2.7 ING4 does not affect activity of HPH-2C *in vitro*.
HIF prolyl hydroxylase activity of recombinant HPH-2C protein was assayed by [^{35}S]-pVHL pull-down assay where immobilized HIF-1α peptide substrate incubated with HPH-2C protein and increasing amounts of recombinant ING4 protein (relative to HPH-2C enzyme) was used to capture [^{35}S]-pVHL protein. Peptide incubated in the absence of HPH-2 enzyme and synthetic hydroxylated peptide served as negative and positive controls for the assay, respectively.

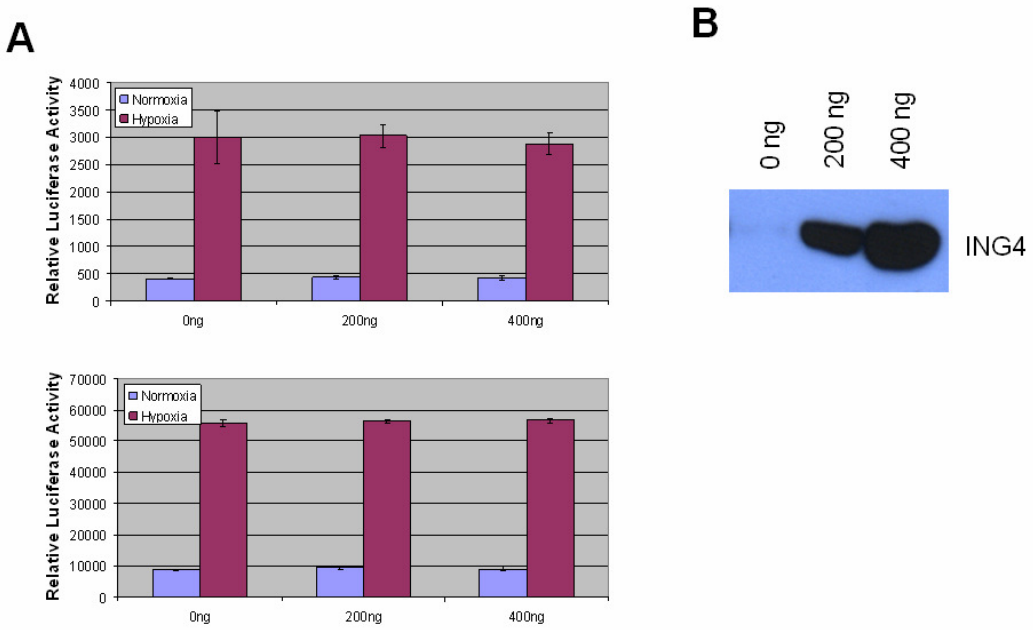


Figure 2.8 Transient overexpression of ING4 does not affect hypoxia reporter gene expression.
(A) HeLa cells stably transfected with 3XHRE-tk-Luciferase (upper panel) or CMV-Luciferase-HIF-1α ODD (lower panel) were transiently transfected with increasing amount of ING4 expression construct and luciferase activity was measured following incubation under normoxic and hypoxic conditions for 15 h. Mean value and standard deviation of triplicate samples are shown.
(B) ING4 protein levels in transfected cells were analyzed by western blot and a representative blot is shown.

stability of HIF-1α protein, which binds to 3XHRE-tk artificial promoter to induce reporter expression, whereas level of constitutively expressed Luciferase-HIF-1α ODD fusion protein is controlled similar to HIF-1α by HPH enzymes due to the presence of a fused ODD. Neither 3XHRE-tk-Luciferase nor CMV-Luciferase-HIF-1α ODD reporters were affected by ING4 overexpression under normoxic or hypoxic conditions. Note that the transfected ING4 was expressed at much higher levels compared to endogenous protein, which was not detectable in vector-transfected cells with the shown exposure of X-ray film to western blot membrane (Fig. 2.8B).

ING4 represses hypoxic induction of HIF target genes

With no observed effect of ING4 protein on the activity of HPH-2 in *in vitro* and *in vivo* by overexpression, we used RNA interference (RNAi) to study the function of ING4-HPH-2 interaction. Small interfering RNA (siRNA) oligos designed for ING4 were successfully used to knockdown ING4 protein levels by ~90% as judged by western blot analysis (Fig. 2.9A). Consistent with the lack of ING4's effect on HPH-2 activity, the HIF-1α protein level was not affected by ING4 knockdown under normoxic or hypoxic conditions. Similarly, ING4 knockdown did not affect HPH-2 protein levels. However, to our surprise, expression of HIF target genes (Semenza 2003) were further upregulated under hypoxic conditions in cells transfected with ING4 siRNA compared to cells transfected with control siRNA against green fluorescent protein (GFP) or luciferase (LUC). Expression of HIF target genes, Nip3 and Adenylate Kinase 3 (AK3) were analyzed by northern blot analysis (Fig. 2.9B) whereas Vascular Endothelial Growth Factor (VEGF), Glucose

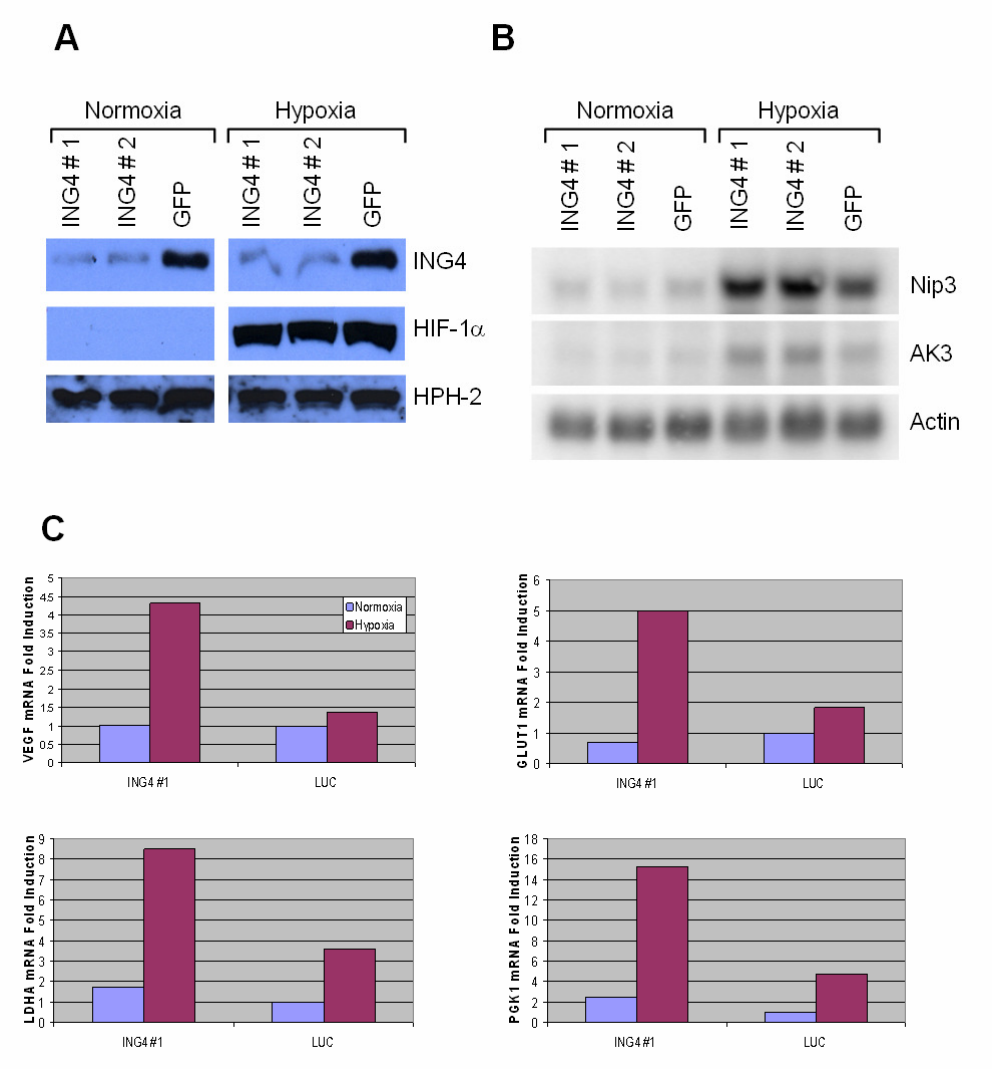


Figure 2.9 Silencing ING4 induces HIF activity under hypoxia.
(A) Levels of ING4, HIF-1α, and HPH-2 proteins in HeLa cells transfected with ING4 (#1 or #2) or GFP siRNA oligos were analyzed by western blot. Transfected cells lysed 3 days after transfection were kept under normoxic conditions except hypoxic exposure for final 15 h of hypoxia samples.
(B) Expression of HIF target genes Nip3 and AK3 were analyzed by Northern Blot in RNA samples prepared from HeLa cells transfected and treated as in *A*. Actin mRNA levels were analyzed to ensure equal loading samples.
(C) Expression of HIF target genes VEGF, GLUT1, LDHA, and PGK1 were analyzed by Quantitative Real-time PCR (qRT-PCR) in RNA samples prepared from HeLa cells transfected with ING4 #1 or Luciferase siRNA oligos and treated as in *A*. mRNA levels for every gene in each sample was corrected with Cyclophilin mRNA levels.

Transporter 1 (GLUT1), Lactate Dehydrogense A (LDHA), and Phosphoglycerate Kinase 1 (PGK1) were analyzed by quantitative real-time PCR (qRT-PCR) (Fig. 2.9C). These genes were found to be induced 2-3 times more upon ING4 knockdown during hypoxia. Because this increase in gene induction was not due to an increase in HIF-1α protein level (Fig. 2.9A), this finding pointed toward a mechanism in which ING4 functions as a hypoxia restricted repressor on HIF transcriptional activity.

ING4 and HPH-2 form a complex with HIF-1α

The hypoxia specific effect of ING4 on expression of HIF target genes suggested that ING4 and HPH-2 together might form a complex with HIF-1α and modulate its transcriptional activity. To address this possibility, we performed a series of experiments. First, [^{35}S]-HIF-1α was pulled down with GST-HPH-2C in the absence of 2-oxoglutarate and ascorbate –where hydroxylase activity of HPH-2 is compromised– to recapitulate the hypoxic interaction between the two. As expected, both HIF-1α and ING4 were efficiently pulled down with GST-HPH-2C but not GST (Fig. 2.10A). Recombinant ING4, HPH-2C, ODD of HIF-1α proteins were purified and resolved on size exclusion column singly or in combination to test for a complex formation (Fig. 2.10B), in a manner similar to that shown in Fig. 2.3C. HIF-1α ODD eluted as a single peak on Superdex 200 column consistent with a monomeric species. However, incubation of HIF-1α ODD with ING4 and HPH-2C shifted the HIF-1α ODD elution profile towards higher molecular weight fractions indicative of formation of a ternary complex containing HIF-1α ODD, HPH-2, and ING4.

Furthermore, we tested whether endogenous ING4 and HPH-2 proteins are associated with Hypoxia Response Element (HRE), through which HIF protein complex mediates expression of its target genes *in vivo,* by chromatin immunoprecipitation experiments. To this end, HeLa cells stably transfected with 3XHRE-tk-Luciferase reporter construct were exposed to hypoxic conditions to induce assembly of HIF complex at 3XHRE promoter and chromatin fragments prepared from these cells were precipitated with ING-4, HPH-2, and ARNT/HIF-β immune sera (Fig. 2.10C). Consistent with the formation of a ternary protein complex with HIF, ING4 and HPH-2 immune sera were able to immunoprecipitate the 3XHRE artificial promoter. Binding of HIF complex was confirmed by immunoprecipitation of the same chromatin fragment with ARNT immune serum. In control experiments, the 3XHRE promoter was not immunoprecipitated with pre-immune serum or Protein A-agarose beads alone (No Ab). The limited efficiency of ING4 and HPH-2 immune sera in precipitating 3XHRE promoter can be attributed to the restricted accessibility of corresponding proteins to antibodies within the ING4-HPH-2-HIF protein complex or non-stoichiometric interaction of ING4 and HPH-2 with the HRE-bound HIF, that is, only a fraction of HRE-bound HIF might be associated with ING4 and HPH-2 *in vivo*. Nevertheless, these data support that under hypoxic conditions, at least a fraction of the endogenous ING4 and HPH-2 proteins are associated with the HIF complex present at HRE-containing promoters.

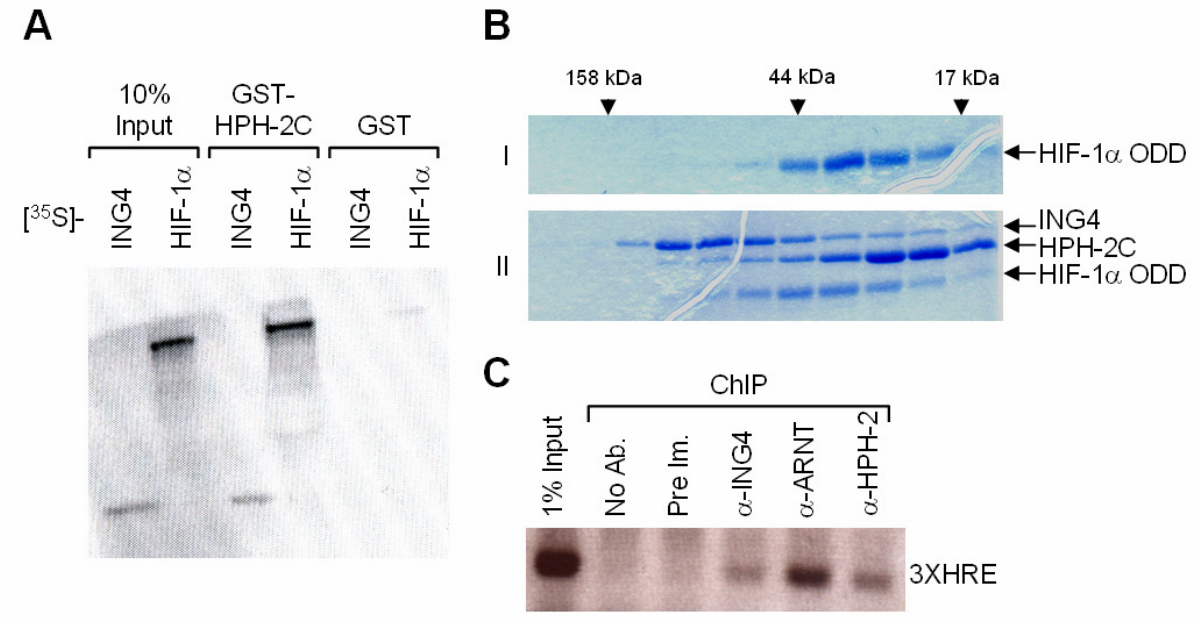


Figure 2.10 ING4, HPH-2, and HIF-1α form a complex at Hypoxia Response Elements (HREs).
(A) Interaction of HPH-2C with ING4 or HIF-1α proteins were assayed by GST pull-down experiment where radiolabeled ING4 and HIF-1α proteins were pulled down with GST-HPH-2C or GST proteins bound to glutathione resin. 10% of each radiolabeled protein used for the assay was shown on the left.
(B) Purified recombinant HIF-1α ODD protein was separated on Superdex 200 size exclusion column alone or mixed with HPH-2C and ING4. Interaction between proteins was assessed by a shift of proteins to higher molecular weight fractions.
(C) Association of ING4 and HPH-2 with HIF bound to Hypoxia Response Elements on chromatin was assayed by chromatin immunoprecipitation assay (ChIP). Chromatin fragments of HeLa 3XHRE-tk-Luciferase stable cell line kept under hypoxic conditions for 15 h were immunoprecipitated with pre-immune serum (negative control), ING4, HPH-2, and ARNT (positive control) specific antibodies or without any antibody (No Ab.). 3XHRE promoter from each precipitation and 1% of the lysate used for ChIP were amplified by PCR.

ING4 suppresses HIF activity in a chromatin-dependent manner

ING family proteins are nuclear proteins with mostly chromatin-related functions. They have been shown to associate with histone acetyltransferase and/or histone deacetylase complexes to alter chromatin structure, therefore regulating transcriptional activation, repression, DNA repair, and replication (Feng, Hara et al. 2002). Chromatin immunoprecipitation and RNA interference experiments indicated that ING4 protein was bound to HIF target promoters under hypoxia and modulate HIF transcriptional activity. However, it was not clear whether the repressive function of ING4 was dependent on chromatin structure or the DNA sequence alone. To address this issue, luciferase expression from a stably and transiently transfected 3XHRE-tk-Luciferase construct was assayed after suppression of ING4. In stably transfected HeLa cells luciferase activity was induced ~10 fold by hypoxic exposure of GFP control siRNA treated cells (Fig. 2.11). Similar to its effect on endogenous HIF target genes, suppression of ING4 further induced luciferase reporter expression by ~2 fold under hypoxia without any significant effect under normoxia, which is both qualitatively and quantitatively consistent with data represented in Fig. 2.9B and 2.9C. Similar results were obtained with multiple clones of HeLa 3XHRE-tk-Luciferase stable cell line eliminating the possibility of an artifactual result (data not shown). The transiently transfected reporter construct was inducible by hypoxia, though to a lesser extent (~3 fold) due to high basal expression under normoxia. Interestingly, luciferase reporter expression was not sensitive to ING4 suppression when transiently transfected indicating that ING4 regulates HIF transcriptional activity in a chromatin-dependent manner.

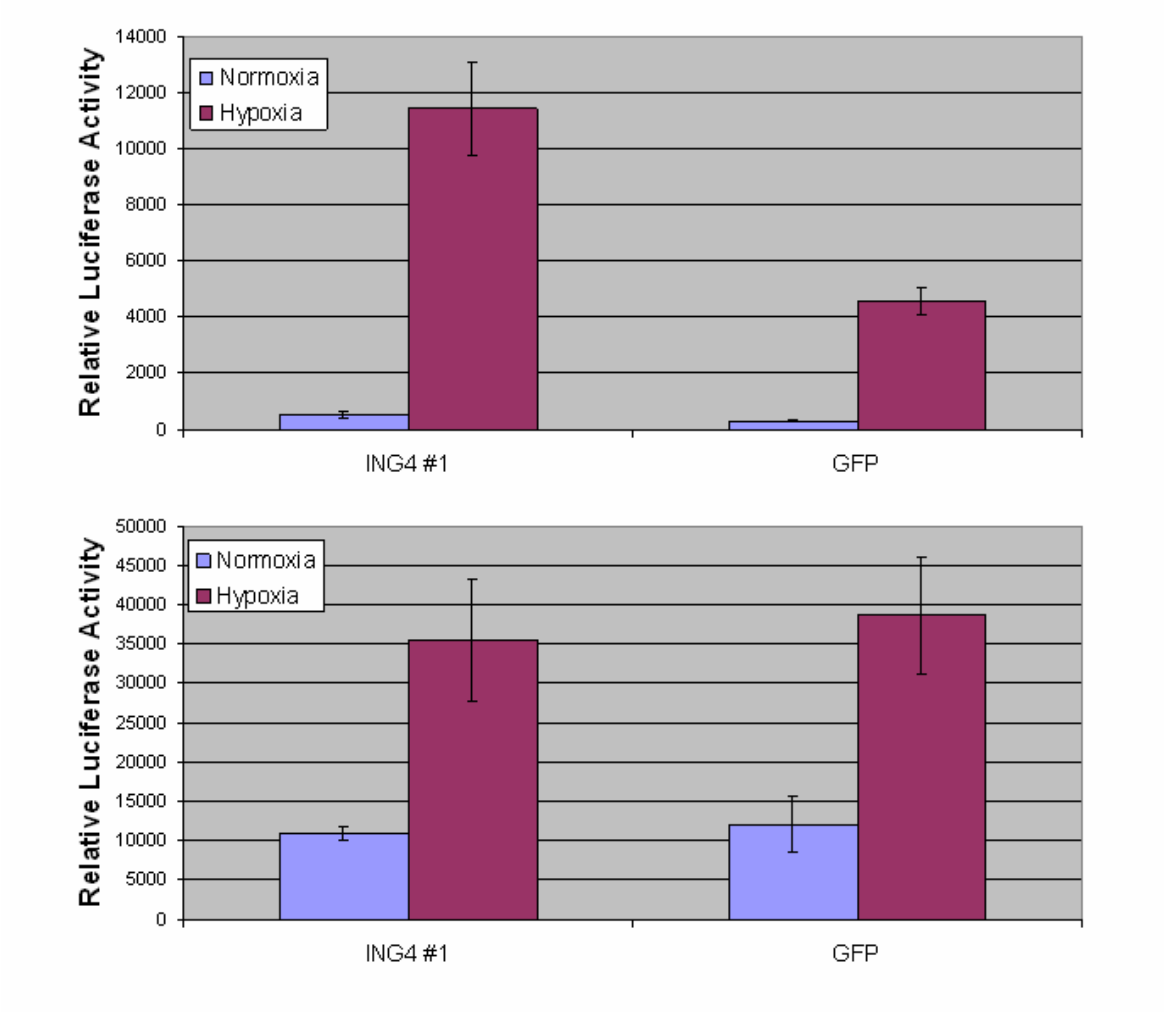


Figure 2.11 Silencing ING4 induces HIF activity in a chromatin-dependent manner.
HeLa cells either stably (upper panel) or transiently transfected (lower panel) with 3XHRE-tk-Luciferase reporter construct were transfected with ING4 #1 or GFP siRNA oligos. Luciferase activity of cells that were kept under normoxia except for exposure of hypoxia samples to 1% O_2 for 15 h was measured 3 days after transfection. A representative example for several stable cell lines with similar results is shown. Mean value and standard deviation of triplicate samples are shown.

Discussion

Identification of HIF Prolyl Hydroxylases (HPHs) (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001; Ivan, Haberberger et al. 2002) and HIF asparaginyl hydroxylase (FIH-1) (Hewitson, McNeill et al. 2002; Lando, Peet et al. 2002) has advanced our understanding of how HIF transcription factor, the master regulator of cellular hypoxia response pathway, is regulated in response to oxygen concentration. Although oxygen serves as the main regulator of HIF hydroxylase activity, HIF hydroxylases are likely to be subjected to regulation by signaling pathways, posttranslational modifications, feedback loops, subcellular localization, and protein–protein interactions. In an effort to identify such HPH interacting proteins, a yeast two-hybrid screen had been performed using C-terminal hydroxylase domain of HPH-2 (HPH-2C) as bait from which ING4, which belongs to a relatively small protein family known as Inhibitor of Growth (ING) containing five members in mammals (ING1-5) (Feng, Hara et al. 2002), and several other proteins had been identified (Chapter 1).

This yeast two-hybrid interaction between ING4 and HPH-2C was further evaluated with several additional methods (Fig. 2.3) and shown to be mediated through the PHD of ING4 (Fig. 2.4). Unlike the GFP-HPH-2 fusion protein that localized exclusively in cytoplasm (Metzen, Berchner-Pfannschmidt et al. 2003), we showed that a substantial fraction of endogenous HPH-2 protein was localized in nucleus together with ING4 (Fig. 2.5) allowing these two proteins to form a nuclear complex *in vivo*. We initially predicted that the significance of the HPH-2-ING4 interaction would be manifested as; i) ING4 acts as a HPH-2 substrate similar to HIF-α ODD fragments identified in the same two-hybrid screen, or ii)

ING4 interacts with HPH-2 to modulate its hydroxylase activity. In the former case, the possibility of ING4 acting as a HPH-2 substrate was remote, since ING4 protein had no sequence similarity to conserved LXXLAP hydroxylation motif shared by the known HPH substrates (Huang, Zhao et al. 2002). Furthermore, *in vitro* studies did not detect any HPH-2C activity against recombinant ING4 by a [^{14}C]-2-oxoglutarate decarboxylation assay (Fig. 2.6) and mass spectrometry (data not shown). In the latter case, we did not detect any effect of ING4 on HPH-2 activity (Fig. 2.7). Evidently, the precise function of ING4 is different than that of OS-9, a recently identified HPH-2 interacting protein (Baek, Mahon et al. 2005). OS-9, like ING4, forms a complex with HPH-2 and HIF-1α but, unlike ING4, OS-9 promotes hydroxylation and degradation of HIF-1α.

Interestingly, suppression of ING4 enhanced HIF transcriptional activity under hypoxia measured by expression of endogenous HIF targets (Fig. 2.9B and 2.9C) and stably transfected HIF-dependent luciferase reporter (Fig. 2.10). Qualitative and quantitative similarity (~2-3 fold induction with ING4 suppression) observed between regulation of endogenous HIF target and reporter gene expression indicated that 3XHRE-tk-Luciferase reporter construct can be used to monitor HIF activity *in vivo*. Enhancement of HIF transcriptional activity upon ING4 suppression was not due to increase in HIF-1α subunit stability (Fig. 2.9A), which is consistent with the lack of ING4's effect on HPH-2 hydroxylase activity *in vitro* (Fig. 7). Although HIF-2α was not detected under these conditions in HeLa 3XHRE-tk-Luciferase stable cell lines, we still can not exclude the possibility that HIF-2α and -3α might account at least in part for the induced HIF activity. ING4 overexpression on the other hand did not have any effect on HIF activity as measured

by luciferase expression in HeLa 3XHRE-tk-Luciferase stable cell line (Fig. 2.8). Furthermore, the repressive effect of ING4 was dependent on chromatin structure (Fig. 2.11). Luciferase reporter was further induced upon ING4 suppression only when the reporter construct was stably integrated in genome but not when it was transiently transfected.

We also demonstrated that ING4, HPH-2, and HIF-1α form a nuclear complex. *In vitro*, purified recombinant ING4, HPH-2, and HIF-1α ODD proteins form a ternary complex as indicated by a shift to larger molecular weight fractions in size exclusion chromatography (Fig. 2.10B). It is not clear whether trimerization of recombinant ING4 protein (Fig. 2.3C) is an *in vitro* artifact or it serves a biological function *in vivo*. Chromatin immunoprecipitation experiments revealed that both endogenous ING4 and HPH-2 proteins are associated with hypoxia response elements recognized by HIF under hypoxic conditions (Fig. 2.10C).

Based on our data, we propose the following model for regulation of HIF transcriptional activity by ING4 (Ozer and Bruick 2005) (Fig. 2.12). Under hypoxic conditions HIF-α subunit heterodimerizes with its obligate partner ARNT and binds to hypoxia response elements (HREs) in the promoters of target genes. HIF-α subunit through its C-terminal Transactivation Domain (CTAD) then recruits transcriptional coactivators such as CBP/p300 to induce gene expression. HPH-2 bound to the Oxygen-dependent Degradation Domain (ODD) of HIF-α then further recruits transcriptional repressors associated with ING4 to inhibit HIF transcriptional activity and identification of such repressors will reveal the details about the connection between HIF repression and ING4 complex. Limited accessibility of ING4 and HPH-2 proteins within such a complex would be consistent with inefficient immunoprecipitation of 3XHRE-containing chromatin fragment

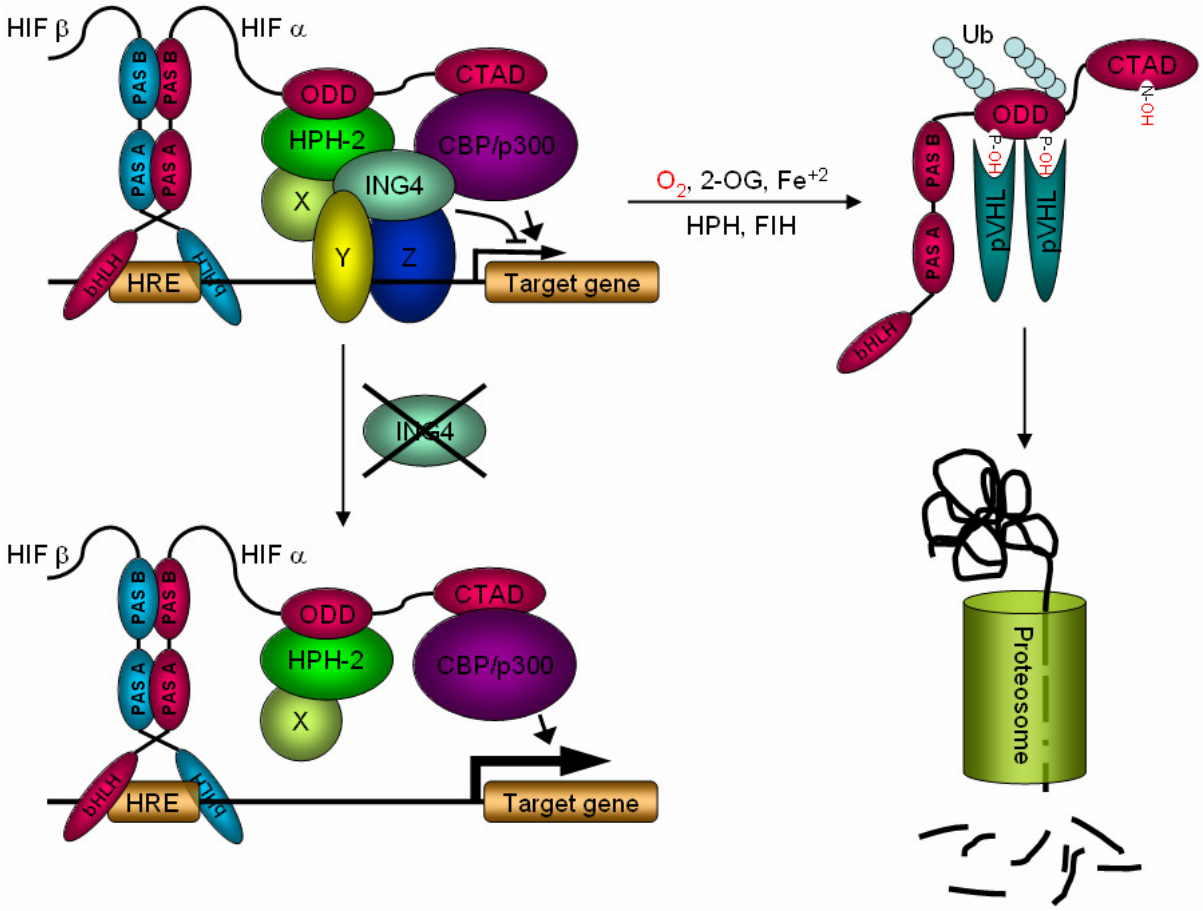


Figure 2.12 Model for ING4 function in hypoxia response pathway.
HIF-α subunit is hydroxylated by FIH-1 and HPH enzymes at C-terminal Transactivation Domain (CTAD) and Oxygen-dependent Degradation Domain (ODD) when oxygen levels are high to block recruitment of CBP/p300 transcriptional coactivators and to target α subunit for proteosomal degradation, respectively. When oxygen concentrations are limiting, heterodimeric HIF complex binds hypoxia response elements (HREs) in target gene promoters where it recruits transcriptional coactivators to induce gene expression. HPH-2 inactivated in the absence of oxygen binds to its substrate, HIF-1α ODD, and recruits ING4 and associated transcriptional repressors to modulate HIF transcriptional activity. Reduction in ING4 protein levels as seen in some cancer cells will induce HIF target genes which may contribute to tumor formation and growth. Figure and figure legend are taken from (Ozer and Bruick 2005).

with ING4 and HPH-2 immune sera (Fig. 2.10C). A constitutive interaction between HPH-2 and HIF-α ODD under hypoxia also allows HPH-2 to hydroxylate and target HIF-α for proteosomal degradation as soon as oxygen becomes available.

We believe that regulation of HIF activity by recruitment of ING4 complex is not the major mode of regulation; instead, it functions as a fine-tuning mechanism to modulate the magnitude of HIF transcriptional activity. It is not clear whether every HIF target gene is regulated by ING4 in a similar manner even though all the HIF targets that we tested were upregulated in response to ING4 suppression with similar magnitudes, the possibility of gene specific effects of ING4 can not be ruled out. Our model is consistent with prior literature and even offers explanations to some observations noted earlier. For example, stable interactions between HPH-2 and HIF-α ODD under hypoxia can explain the short half-life (~15 minutes) of HIF-α subunit upon re-oxygenation (Jewell, Kvietikova et al. 2001). As integrated in our model, regulation of HIF transcriptional activity by recruitment of transcriptional repressors via ING4 is a novel function of HPH-2 enzyme in addition to control of HIF-α stability. This novel function of HPH-2 has been reported by others as well(To and Huang 2005); however, their proposed mechanism, blockage of HIF-α NTAD interaction with yet unknown transcriptional co-activator(s) by binding of HPH-2 to the overlapping ODD, differs from ours, recruitment of ING4 containing transcriptional co-repressor complex to HIF by HPH-2.

Considering the extent of sequence similarity between PHDs, a positive interaction between other ING family proteins and HPH-2C was expected and experimentally shown by GST pull down experiments (Fig. 2.3B). In addition, our preliminary results with suppression

of expression using siRNA oligos (data not shown) suggest that other ING family members may also participate in regulation of HIF activity. Likewise, HPH-1 and -3 proteins can be expected to interact with ING family proteins due to similarity between hydroxylase domains of the three HPH enzymes. These potential combinatorial interactions need to be investigated in detail in future, because it is possible that some of these interactions might serve redundant or even opposing biological functions.

ING4 has been reported to function in several other cellular pathways. For example, a repressor function of ING4 has been reported against the NF-κB transcription factor (Garkavtsev, Kozin et al. 2004) as seen in glioblastoma cells where reduced expression levels of ING4 have been correlated with upregulation of angiogenic NF-κB targets such as IL-6, IL-8, and COX-2; this elevated gene expression was repressed by transfection of exogenous ING4. It is therefore clear that NF-κB represents yet another transcription factor which is modulated by ING4. Enhanced angiogenesis observed by Garkavtsev and his colleagues in glioblastoma tumor samples and tumor xenograft models with reduced ING4 levels might at least in part caused by induction of angiogenic targets of HIF. One such HIF target gene, VEGF, was not induced in glioblastoma cells with reduced ING4 levels (Garkavtsev, Kozin et al. 2004); however, it should be noted this experiment was conducted under the normoxic conditions where one would not expect HIF-α to accumulate and ING4 to alter HIF transcriptional activity.

Further work will be required to clarify details of our model and it will be complicated because of the possible redundancy between ING family members, HPH enzymes, and HIF-α subunits. Moreover, it is already known that some ING family members

can be part of multiple protein complexes (Feng, Hara et al. 2002) which might even have opposite function as seen with ING1 (Nourani, Howe et al. 2003). Taken all together, these protein-protein interactions will determine the function and target specificity of ING4 complex. Mechanistic understanding how ING4 functions as a transcriptional repressor for HIF, NF-κB, and perhaps other transcription factors will give insight into the regulation of several key cellular pathways, and this understanding is dependent on identification of ING4 associated proteins.

CHAPTER THREE
Identification of ING4 interacting proteins: Understanding the mechanism of ING4 function

Introduction

Though they have been implicated in a variety of cellular processes such as transcriptional regulation, cell cycle control, proliferation, and apoptosis (Feng, Hara et al. 2002), ING family proteins are simple in terms of their domain organizations. While PHD-type zinc fingers, which are almost exclusively found on chromatin-related proteins (Aasland, Gibson et al. 1995), and nuclear localization signals are invariable features of ING family members, only a few other motifs such as the leucine zipper-like (LZL) motif are present in certain members of ING family (Fig. 2.2). To date no enzymatic activity of ING family proteins has been identified. However, ING family proteins purified from yeast associate with histone acetyltransferase (HAT) activity (Loewith, Meijer et al. 2000) while mammalian ING proteins reside in both HAT and histone deacetylase (HDAC) complexes (Skowyra, Zeremski et al. 2001; Vieyra, Loewith et al. 2002), where they are thought to act as regulatory components. Recently, PHD zinc fingers of ING proteins were found to recognize a specific subset of phosphoinositides (PtdInsPs) (Gozani, Karuman et al. 2003) and methylated lysine residues (Mellor 2006; Zhang 2006). Therefore, ING family proteins are believed to function as an interface between signaling events and downstream transcriptional responses thereby alter the aforementioned cellular processes.

ING4, identified based on sequence similarity with ING1, was shown to inhibit cell growth and induce apoptosis in a p53-dependent manner (Shiseki, Nagashima et al. 2003).

ING4 induced expression from p21 –a p53 target– promoter and acetylation of p53. Physical interaction between ING4 and transcriptional co-activator histone acetyltransferase p300 provided some explanation for the link between ING4 and p53 acetylation/activation (Shiseki, Nagashima et al. 2003). In addition to p53, activities of HIF (Ozer, Wu et al. 2005) (Chapter 2) and NF-κB (Garkavtsev, Kozin et al. 2004) transcription factors were also shown to be regulated by ING4 but instead negatively. Garkavtsev and his colleagues have analyzed expression profiles of about 300 angiogenesis-related genes in cell culture models of ING4 (overexpressed or repressed) following their initial observation that expression level of ING4 inversely correlates with vascularization and growth of brain tumors. A repressive effect of ING4 was observed on expression of well known NF-κB target genes; IL6, IL8, and COX2 (Garkavtsev, Kozin et al. 2004). Furthermore, ING4 was shown to interact with the p65 subunit of NF-κB transcription factor suggesting a direct regulation of NF-κB activity; however, the underlying mechanism of this transcriptional repression was not elucidated. Even though ING4's interaction with transcriptional coactivator p300 is consistent with activation of p53, it contradicts with transcriptional repression of HIF and NF-κB both of which are known to be activated by p300 (Arany, Huang et al. 1996; Perkins, Felzien et al. 1997).

The ability of ING4 to regulate transcription either positively or negatively was intriguing since ING4 itself is devoid of any known enzymatic activity. However, based on prior literature about transcriptional regulation of p53 (Laptenko and Prives 2006), HIF (Ruas and Poellinger 2005), and NF-κB (Hoffmann, Natoli et al. 2006), and ING family proteins (Feng, Hara et al. 2002; Campos, Chin et al. 2004; Shi and Gozani 2005; Russell,

Berardi et al. 2006), one could speculate on how ING4 might inhibit HIF and NF-κB. One possibility is that, similar to ING1 (Vieyra, Loewith et al. 2002), ING4 can be a component of a HDAC-containing co-repressor complex, which when recruited to target promoters alters the chromatin structure making them less accessible to the transcriptional machinery. In an alternate model, similar to ING3 (Doyon, Selleck et al. 2004), ING4 might be a component of a HAT-containing co-activator complex in which ING4 represses transcriptional activation by inhibiting HAT activity. p300 is a candidate histone acetyltransferase for such a model since interaction between ING4 and p300 has been reported (Shiseki, Nagashima et al. 2003) but the functional consequence of this interaction with regards to p300 HAT activity is not known. In a third model, ING4 associated proteins (either HAT or HDAC complexes) might affect the activity of transcriptional factor by altering its acetylation status. Consistent with this model, ING4 was shown to induce acetylation of p53 (Shiseki, Nagashima et al. 2003) and activity of NF-κB has long known to be regulated by acetylation –inhibited or activated depending on the site being modified (Perkins 2006). Furthermore, based on interactions of HIF with multiple HAT enzymes (i.e. CBP, p300 (Arany, Huang et al. 1996), SRC-1, and TIF2 (Carrero, Okamoto et al. 2000)) it was speculated that HIF itself might also be a target for acetylation (Wenger 2002); however, direct evidence for such a regulatory mechanism is lacking.

In order to further understand the mechanism of ING4 function, a better knowledge of ING4 interacting proteins would be fruitful. Identification of additional protein-protein interactions of ING4 will no doubt shed light on how it regulates transcription and subsequently cell cycle, proliferation, and apoptosis. To this end, we pursued identification of

ING4 interacting proteins with multiple methods including yeast two-hybrid screen, biochemical purification, and Tandem Affinity Purification (TAP) (developed by Seraphin group at EMBL, Germany (Rigaut, Shevchenko et al. 1999)). The TAP method consists of sequential immuno-affinity purification steps and analysis of multiple protein-tags showed a general applicability of Protein A domain-TEV protease cleavage site-Calmodulin binding peptide combination (now being referred to as TAP-tag) for purification of protein complexes from various sources including mammalian cells under physiological conditions (Puig, Caspary et al. 2001). Purification of TAP-tagged proteins that are expressed at levels comparable to the endogenous protein increases the likelihood of purifying endogenous complexes as in biochemical purification of the same protein, whereas false-positives due to overexpression are a major concern.

Using these approaches, we identified Breast cancer Metastasis Suppressor 1 (BRMS1) in yeast two-hybrid screen and MYST2/HBO1 and JADE3/PHF16 in TAP method as ING4 interacting proteins. BRMS1 is a component of mSin3A/ING1/HDAC1/2 complex (Meehan, Samant et al. 2004; Nikolaev, Papanikolaou et al. 2004), while MYST2 is a member of MYST family of histone acetyltransferases, which was shown to repress activities of androgen receptor (Sharma, Zarnegar et al. 2000) and NF-κB (Contzler, Regamey et al. 2006).

Experimental Procedures

Cell Culture: Transient Transfections and Luciferase Assay

HeLa and 293 cell lines were grown in Dulbecco's modified Eagle's medium (HyQ DME, HyClone) containing high glucose supplemented with 10% FBS (Gemini Biological Products) whereas U87MG cells were grown in same media further supplemented with 1 mM pyruvate (HyClone) and MEM non-essential amino acids (GIBCO) under normal cell culture conditions 5% CO_2 at 37 °C. Cells were maintained under hypoxic conditions at 37 °C within a humidified hypoxic chamber (Coy Laboratory Products) filled with 1% O_2, 5% CO_2, and 94% N_2 air for 15 h unless indicated otherwise. Transient overexpression of proteins were achieved by transfection of mammalian expression constructs to HeLa or HeLa 3XHRE-tk-Luciferase stable cell line with Lipofectamine/Plus reagent (Invitrogen) according to manufacturer's protocol or to 293 cells with $CaPO_4$ precipitation method. Lipofectamine/Plus reagent was used for transfection of HeLa cells with CMV-driven mammalian expression plasmids using; empty pcDNA3.1/V5-HisA plasmid or containing N-terminal TAP- and 3XFLAG-tagged ING4 (NTAP-3XFLAG-ING4: two tandem repeats of Protein A-TEV protease cleavage site-Calmodulin binding peptide-3XFLAG peptide-ING4). Construction of NTAP-3XFLAG-ING4 was adapted from (Doyon, Selleck et al. 2004). U87MG cells transfected with ING4 pcDNA3.1/V5-HisA, MYST2 p3XFLAG-CMV-10 constructs or empty vectors using Lipofectamine/Plus reagent (Invitrogen). For generation of stable cell lines, transfected cells were selected with 300 μg/ml G418 containing media for 2 weeks. Cell clones were tested by western blot for expression of NTAP-3XFLAG-ING4, ING4 and 3XFLAG-MYST2. For U87MG-ING4 stable cell lines, PCR amplification of transfected ING4 was done to ensure stable integration of the construct using genomic DNA as template and a specific primer pair, one oligo complementary to ING4 and the other to

vector sequence. NF-κB-Luciferase reporter construct was a generous gift of Z. Chen, UT Southwestern Medical Center at Dallas.

siRNA Transfections

All of the duplex siRNA oligos were purchased from Dharmacon as pre-annealed and deprotected form (A4 option). siRNA transfections and luciferase assays were performed as described in Chapter 2. Sequences of oligos were: BRMS1 #1 AGAGCUCCGAGAUGGAUGAdTdT and UCAUCCAUCUCGGAGCUCUdTdT; BRMS1 #2 GGAAUAAGUACGAAUGUGAdTdT and UCACAUUCGUACUUAUUCCdTdT; MYST2 #1, AGAGGCAGCUUCGAUAUAAdTdT and UUAUAUCGAAGCUGCCUCUdTdT; MYST2 #2, UGAGGAGCCUGCUUACUCUdTdT and AGAGUAAGCAGGCUCCUCAdTdT; MYST2 #1a, GAAAUGCGCCUUCUUCUGAdTdT and UCAGAAGAAGGCGCAUUUCdTdT; MYST2 #1b, GCUGUAACUCUCUAGGACAdTdT and UGUCCUAGAGAGUUACAGCdTdT.

Yeast Two-Hybrid Screen

S.cerevisiae Y187 strain (MATα, ura3, his3, ade2, trp1, leu2, gal4Δ, gal80Δ, met$^-$, URA3 : : $GAL1_{UAS}$-$GAL1_{TATA}$-lacZ MEL1) pretransformed with human fetal brain cDNA library in pACT2 vector was purchased from Clontech. cDNA encoded proteins were expressed as GAL4 activation domain (GAL4 AD) fusion proteins from pACT2 vector containing LEU marker. ING4 protein encoding sequence was cloned into pGBKT7 vector (Clontech) containing TRP marker to express GAL4 DNA binding domain-ING4 fusion

protein. *S.cerevisiae* AH109 strain (MATa, trp1, leu2, 112, ura3, his3, gal4Δ, gal80Δ, LYS2 : : $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3 : : $MEL1_{UAS}$-$MEL1_{TATA}$-lacZ, MEL1) transformed with ING4-pGBKT7 bait construct using LiAc transformation method was mated to pretransformed Y187 cells, plated on medium stringency synthetic defined medium (2% glucose, 0.67% nitrogen base without amino acids (Difco) supplemented with SD Medium–LEU–TRP–HIS (Q-BIOgene)), and incubated at 30 °C for 2 weeks. Colonies were assayed by growth on high stringency media (–LEU–TRP–HIS–ADE) as well as by X-gal staining for LacZ reporter gene expression. Plasmids were extracted using Zymoprep yeast plasmid miniprep kit (Zymo Research), retransformed into AH109 cell containing ING4-pGBKT7 or pGBKT7 and re-assayed. Plasmids that allowed yeast to grow on SD–LEU–TRP–HIS–ADE medium and induce LacZ expression only when co-transformed with ING4-pGBKT7 construct but not with pGBKT7 vector were sequenced to identify the genes. Positive cDNA clones were subcloned from pACT2 vector together with HA-tag into pcDNA3.1/V5-HisA mammalian expression vector for follow up experiments.

Antibodies and Western Blot Analysis

Polyclonal antiserum was obtained from rabbits (two per protein) immunized with HPH-2C, ING4, BRMS1, or HIF-β/ARNT (a. acids 1-140) recombinant proteins. Mouse monoclonal antibodies to HA.11-epitope (Covance Research), FLAG-epitope (Sigma-Aldrich), and HDAC2 (Santa Cruz Biotechnology), and goat (MYST2 Ab#1, Santa Cruz Biotechnology) and rabbit polyclonal antibodies to MYST2 (MYST2 Ab#2, Abcam) were

purchased from indicated companies. Cells were lysed with SDS sample buffer, resolved by SDS PAGE and transferred to Hybond C-extra membrane for Western Blot analysis. Primary antibodies were detected by species-specific HRP-conjugated secondary antibodies (Jackson ImmunoResearch) with enhanced chemiluminescence (Amersham Biosciences).

Recombinant ING4 protein coupled to AminoLink Coupling gel (Pierce) according to manufacturer's protocol was used to affinity purify ING4 anti-serum. ING4-coupled gel washed with 10 column volumes (CV) of the following buffer: 10 mM Tris.Cl pH 7.5; 100 mM Glycine pH 2.5; 10 mM Tris.Cl pH 8.8; freshly prepared 100 mM Ethylamine pH 11.5; and 10 mM Tris.Cl pH 7.5. Antiserum diluted 1:10 with 10 mM Tris.Cl pH 7.5 was passed over gel 5 times. Antibody-bound gel was washed with 20 CV of 10 mM Tris.Cl pH 7.5 and 500 mM NaCl, 10 mM Tris.Cl pH 7.5 buffers. Bound antibody was eluted first with 10 CV of 100 mM Glycine pH 2.5 and then 10 CV of 100 mM Ethylamine pH 11.5 into 1 CV of 1 M Tris.Cl pH 8.0 each time with a wash step (10 CV of 10 mM Tris.Cl pH 8.0) in between two elutions. Two elutions were combined and dialyzed against 1X PBS containing 0.05% NaN_3 and stored at -80 °C as aliquots.

Co-immunoprecipitation

HeLa or 293 cells transfected with indicated mammalian expression constructs were lysed with 20 mM Tris.Cl pH 7.5, 100 mM NaCl, 5 mM 2-mercaptoethanol, 1% NP-40, and Protease Inhibitor Cocktail (Sigma) 24-48 h post-transfection. Transfected cell lysates or HeLa 0.4 M KCl nuclear extracts incubated in the presence of preimmune serum or immune serum/antibody overnight at 4°C. Antibody-protein complexes were precipitated with Protein

G-agarose beads (Roche Diagnostics), washed extensively with lysis buffer, and eluted with SDS sample buffer. For immunoprecipitation experiments done with HeLa cell nuclear extracts, Buffer A was used to wash the beads. Co-immunoprecipitated proteins were detected by Western blotting using indicated specific antibodies. p65 NF-κB expression plasmid was a generous gift of C. Mendelson, UT Southwestern Medical Center at Dallas.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitations were done as described in Chapter 2. Primer pairs used for amplifications of 3XHRE (227 bp), COX2 (305), and IL6 (219) promoters were as follows: 3XHRE, 5'-GTGCAGGTGCCAGAACATT-3' and 5'-CGGTAGGTCGAGAGGTCAGA-3'; COX2(Nie, Pang et al. 2003), 5'-AAGACATCTGGCGGAAACC-3' and 5'-ACAATTGGTCGCTAACCGAG-3'; and IL6, 5'-ACGACCTAAGCTGCACTT-3' and 5-'GCAGAATGAGCCTCAGAC-3'.

HeLa S100 Cytoplasmic and Nuclear Protein Fractionation

HeLa S3 cell pellet from 50-100 liter culture was purchased from National Cell Culture Center (Biovest International). Cell pellet was resuspended in 4 packed cell volume (PCV) of Buffer A (20mM HEPES-KOH pH 7.5, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA) supplemented with fresh 0.1 mM PMSF and Protease Inhibitor Cocktail and incubated on ice for 20 min. Cells lysed with 50 strokes in dounce homogenizer were centrifuged at 5,000 rpm at 4°C for 30 min. Supernatant was centrifuged at 100,000xg at 4°C for 1 hour. Supernatant from 100,000xg spin was aliquoted and stored at -80 °C as

S100 cytoplasmic fraction. Pellets from 5,000 rpm spin were resuspended in 3 PCV of Buffer A containing 400 mM KCl, dounce homogenized (10 strokes), and incubated on ice for 20 min with gentle stirring to extract nuclear proteins. Nuclear extract was centrifuged at 15,000 rpm in Beckman JA-20 rotor at 4°C for 20 min. Pellets were re-extracted with Buffer A containing 1M KCl. Both 0.4M and 1M KCl nuclear extracts were dialyzed against Buffer A and stored at -80 °C following final 15,000 rpm (Beckman JA-20 rotor) at 4°C for 30 min spin.

Biochemical Purification of ING4

Nuclear proteins of Hela S3 cells (0.4 M KCl extract) were precipitated with ammonium sulfate. 1.314 g ammonium sulfate salt was added slowly to 10 ml extract (24% saturation), mixture was centrifuged at 16,000xg for 15 min at 4 C and 0.976 g of ammonium sulfate (40% saturation) was added to supernatant and centrifuged as before. Protein pellet (24-40% saturation) resuspended in 10 ml of 20 mM Tris.Cl pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.2 mM PMSF was dialyzed against the same buffer with multiple changes to remove ammonium sulfate salt and applied to HiTrap Q column (Amersham Biosciences) in two separate runs. Using the same buffer bound proteins were eluted with linear gradient of NaCl reaching 1 M in 20 column volumes. ING4 containing fractions as determined by western blot analysis were combined, diluted with 7 volumes of 20 mM Tris.Cl pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM β-mercaptoethanol, applied to HiTrap DEAE column (Amersham Biosciences), and eluted with NaCl gradient as done for Q column. ING4 containing fractions were pooled, diluted with 15 volumes of 10 mM $K.PO_4$

pH 8.0, 10 mM KCl, 5 mM β-mercaptoethanol, and applied to CHT II hydroxyapatite column (BioRad). Bound proteins were eluted with gradient of $K.PO_4$ reaching 500 mM concentration in 20 column volumes. Fractions containing ING4 were pooled, dialyzed against 20 mM $K.PO_4$ pH 6.6, 10 mM KCl, 5 mM β-mercaptoethanol with multiple changes of buffer, and applied to HiTrap SP column (Amersham Biosciences). Bound proteins were eluted in the same buffer with linear gradient of NaCl reaching 1 M in 20 column volumes. ING4 containing fractions were dialyzed against 200 mM $K.PO_4$ pH 6.6, 10 mM KCl, 5 mM β-mercaptoethanol, 0.3% NP-40 and immunoprecipitated with affinity purified ING4 antibody and Protein G-agarose beads. Immunoprecipitated proteins and HiTrap SP column fractions were resolved by SDS PAGE and silver stained.

Tandem Affinity Purification (TAP) of ING4

HeLa stable cell line expressing N-terminal TAP-tagged ING4 was grown to 90-100% confluence in 25 15cm plates under normoxic conditions. Cells were collected by trypsin treatment and nuclear extracts were prepared from cell pellet washed twice with ice-cold PBS. Cells were resuspended in 5 packed cell volume of Buffer A (20 mM Tris.Cl pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM DTT, 0.4 mM PMSF, 1 mM Na_3VO_4, 1:200 Protease Inhibitor Cocktail). Cells were lysed by passage through a 25-gauge needle and centrifuged at 10,000 rpm for 10 min at 4 °C. The cytosolic supernatant was removed, and the nuclear pellets were resuspended in 4 volumes of Buffer C (20 mM Tris.Cl pH 7.5, 0.42 M KCl, 1.5 mM $MgCl_2$, 2 mM DTT, 0.4 mM PMSF, 20% glycerol, 1 mM Na_3VO_4, 1:200 Protease Inhibitor Cocktail), and incubated at 4 °C for 30 min with gentle stirring. The

sample was centrifuged at 18,000xg for 15 min at 4°C to separate the extracted nuclear proteins from insoluble material. Nuclear extract was dialyzed against Buffer D (10% Glycerol, 50 mM HEPES-KOH pH 7.5, 100 mM KCl, 2 mM EDTA, 0.1% NP-40, 2 mM DTT, 10 mM NaF, 0.25 mM Na_3VO_4) and incubated with 100 µl IgG-sepharose resin (Amersham) pre-washed with Buffer D. After overnight incubation on a rotater in cold-room, resins were washed trice with Buffer D and once with TEV Cleavage Buffer (TCB: 10 mM HEPES-KOH pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1% NP-40, 1 mM DTT). Proteins were eluted from resin by 20-24h TEV digestion at 4 °C in 800 µl final volume containing 40 µg recombinant TEV. Following digestion, supernatants were collected and resin were washed twice with 400 µl TCB containing 0.5% NP-40, which were combined with TEV digestion supernatant. Calmodulin binding step was omitted in purification protocol due to inefficient recovery of proteins from resin even with up to 25 mM EDTA. TEV digestion eluate was incubated with 30 µl FLAG M2 agarose resin pre-washed with TCB containing 0.3% NP-40 (TCB-0.3). After 4h incubation on a rotater, resins were extensively washed with TCB-0.3 and proteins were eluted twice with 60 µl TCB-0.3 containing 400 ng/µl 3XFLAG peptide. Combined eluates were centrifuged again to remove residual resin and final eluates were separated on 10% SDS gel for silver staining. Silver staining was done as described in (Shevchenko, Wilm et al. 1996). Briefly, gel fixed in 50% methanol, 5% acetic acid for 20 min, was washed 10 min with 50% methanol, and overnight with ddH_2O changed once after 2 hours. Washed gel was sensitized with 0.02% $Na_2S_2O_3$ for 1 min, washed twice with ddH_2O for 1 min, and stained with ice-cold 0.1% $AgNO_3$ for 20 min. Stained gel was washed twice with ddH_2O for 1 min with change of container in between washes and protein

bands were developed with several changes of fresh 0.04% formalin, 2% Na_2CO_3 solution. Color development was terminated with multiple changes of 5% acetic acid when the staining was sufficient. Specific protein bands were submitted to Protein Chemistry Core Facility, UT Southwestern Medical Center at Dallas, for identification by nano-HPLC/MS/MS.

Results

Identification of ING4 interacting proteins: Yeast two-hybrid screen

Yeast pre-transformed with fetal human brain cDNA library, expressed as GAL4 activation domain fusion proteins, were mated with yeast expressing GAL4 DNA-binding domain (DBD)-ING4 fusion protein. Out of $2.2x10^6$ cDNA clones screened, ~700 primary positive clones were obtained. In a secondary screen, these cDNA-encoded proteins were re-assayed for specific interaction with GAL4 DBD-ING4 but not with GAL4 DBD alone by growth of co-transformed yeast on high stringency selective media and by X-gal staining for LacZ reporter expression. A representative subset of them is shown in Fig. 3.1A. 282 of the 700 initial cDNA clones specifically induced expression of all three reporter genes; ADE and HIS required for growth on selective media and LacZ for β-galactosidase activity. DNA sequences of the 282 true positive hits indicated that 178 of them were actually protein encoding cDNA fragments, which were derived from total of ~50 different genes (Table 3.1). cDNA fragments of genes which have been previously implicated in regulation of transcription (marked by gray shading in Table 3.1) were subcloned into a mammalian expression vector together with the HA-tag for subsequent co-immunoprecipitation

Table 3.1 Positive hits from yeast two-hybrid screen for ING4 interacting proteins.

PROTEIN NAME	ACC. NUM.	Co-exp.	Co-IP
Cell death regulator (GRIM19) protein	NP_057049	+	–
Dynamin1	AAA37318	+	–
Vacuolar ATPase H subunit	NP_998784	+	–
KIBRA protein	NP_056053	+	–
Dynactin isoform 1	NP_004073	+	–
G protein pathway supressor 2	NP_116329		
SWI/SNF ATPase subunit (BRG1)	NP_003063	+	–
Nuclear receptor coactivator, SKI interacting protein	NP_036377	–	
G protein pathway supressor 1	NP_004118	+	+/–
Enhancer of polycomb homolog 2 (EPC2)	NP_056445	–	
mSin3A-associated protein 130 (SAP130)	NP_078821	+	–
Mitogen activated protein kinase 7 (BMK1 Kinase)	NP_620603	–	
Max interacting protein 1 (Mxi1)	NP_569157	–	
Bcl-2 associated transcription factor 1	NP_055554	–	
Breast cancer metastasis suppressor 1	NP_056214	+	+
Atonal homolog 8 (BHLHTF6)	NP_116216	+	–
C10orf78 protein	AAH43256	–	
Nephew of Atonal 3 (BHLHTF)	NP_690862	–	
X-ray radiation resistance associated 1 (XRRA1)	XP_374912	+	–
UMP-CMP kinase	NP_057392	+	–
NADH dehydrogenase (ubiquinone) 1β subcomplex 4	NP_004538	+	–
Neuronal pentraxin II	NP_002514		
Adenosine receptor A2a	NP_000666		
Amyloid beta (A4) precursor like	AAH12889		
AKAP350 splice variant	AAD39719		
Pleckstrin homology-like domain family member1	NP_055972		
Kinesin family member C2 (KIFC2)	NP_665697		
Predicted glycin-piperidine binding protein	XP_375682		
OK/SW-CL.16	BAB93516		
Hypothetical protein FLJ20156	NP_060161		
Alpha II spectrin (SPTAN1)	NP_003118		
Apical protein 2	NP_597713		
Sigma adoptin 1B	XP_217618		
HLA class I histocompatibility antigen α chain H	P01893		
Predicted hypothetical protein	XP_059384		
FLJ13909 protein	AAH08882		
KIAA0068 protein	NP_055423		
NADH dehydrogenase (ubiquinone) 1β subcomplex 9	NP_004996		
Microtubule-associated protein-like protein	NP_060644		
Catenin delta 2 (GT24)	NP_001323		

Spindle pole body protein	AAH46634		
Carboxylesterase 2 isoform 1	NP_003860		
Glycine cleavage system protein H	NP_004474		
KIAA1468 protein	NP_065905		
KIAA1759 protein	NP_115503		
Pericentrin 2	NP_006022		
Hypothetical protein MGC93962	AAH29539		
LOC93622 protein	AAH26970		
HSP70 interacting protein (HSPBP1)	NP_036399		
Tuftelin 1	AAQ88973		

cDNA fragments that were selected for further analysis are highlighted. Detectable co-expression (Co-exp.) and positive co-immunoprecipitation (Co-IP) of individual cDNA fragments with co-transfected 3XFLAG-ING4 are indicated with + sign. Non-expressing clones are indicated with – sign in Co-Exp. column and corresponding Co-IP box is left blank.

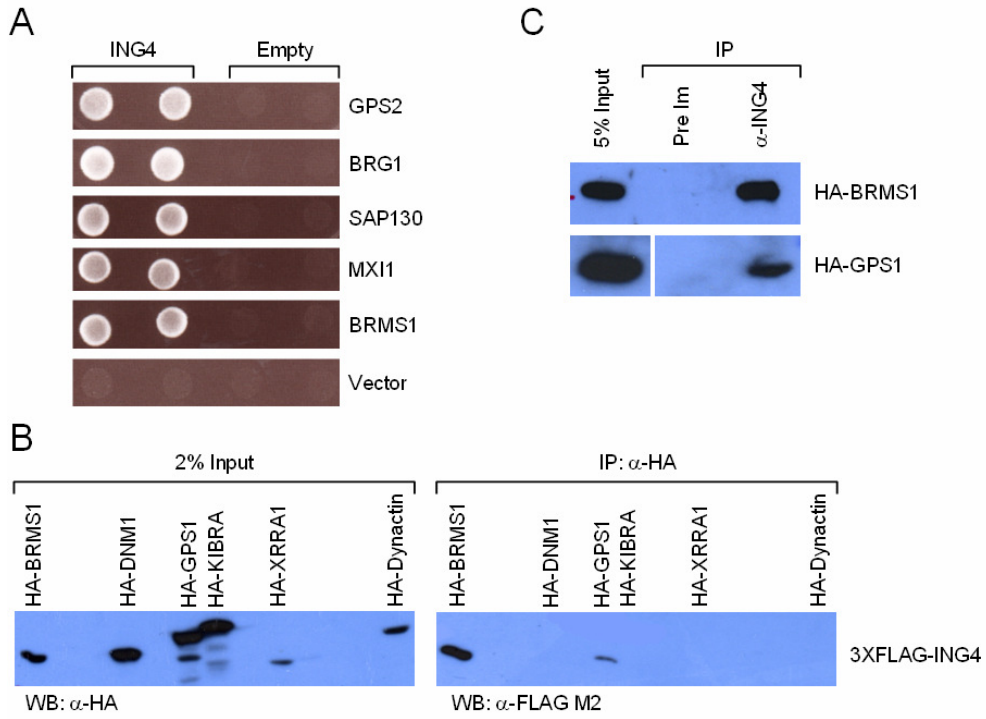


Figure 3.1 ING4 interacts with BRMS1 and GPS1.
(A) Protein-protein interactions of ING4 and yeast two-hybrid screen hits were assayed by growth on –His –Ade –Leu –Trp selective media. AH109 yeast strain re-transformed with ING4 and ING4-interacting protein expressing constructs. Two independent clones for each combination were assayed and corresponding empty vectors were used as negative controls. Only GPS2, BRG1, SAP130, MXI1, and BRMS1 are shown. See Table I for complete list.
(B) Interaction between ING4 and HA-tagged yeast two-hybrid screen positive hits were tested by co-immunoprecipitation.
(C) Interaction between endogenous ING4 and HA-tagged BRMS1 or GPS1 proteins were tested by co-immunoprecipitation. IP, immunoprecipitation; WB, western blotting.

experiments. The HA-tagged proteins co-expressed with 3XFLAG-ING4 in 293 cells were immunoprecipitated with α-HA antibody and co-precipitated 3XFLAG-ING4 protein was detected by western blot analysis using α-FLAG M2 antibody. As shown in Fig. 3.1B, of the expressed proteins only BRMS1 and to a lesser extend GPS1 interacted with ING4. Likewise, in a reciprocal co-immunoprecipitation experiment done in HeLa cells, HA-tagged BRMS1 and again to lesser extend GPS1 were immunoprecipitated together with endogenous ING4 using α-ING4 anti serum (Fig. 3.1C). Neither BRMS1 nor GPS1 were immunoprecipitated with pre-immune serum.

BRMS1 does not affect HIF activity

Due to its stronger interaction with ING4, and its potential involvement in transcriptional control as a component of the mSin3A-histone deacetyltransferase complex (Meehan, Samant et al. 2004), we focused our efforts on BRMS1. Possible regulatory effects of BRMS1 were studied in HeLa 3XHRE-tk-Luciferase reporter cell line. Overexpression of BRMS1 had no effect on the HIF-dependent luciferase reporter either at hypoxic or normoxic conditions (data not shown). Likewise, expression of the luciferase reporter was not affected in cells transfected with BRMS1 siRNA oligos, compared to cells transfected with GFP siRNA, while transfection of ING4 siRNA oligo induced luciferase reporter expression under hypoxia (Fig. 3.2A). Even though both BRMS1 siRNA oligos led to a marked reduction in BRMS1 protein level as determined by western blot analysis (Fig. 3.2B), it is possible that the remaining BRMS1 protein is sufficient enough to maintain its function. Therefore, we

can not completely rule the possible involvement of BRMS1 in regulation of HIF and it requires further study with more efficient silencing of BRMS1.

ING4 does not interact with HDAC2

Lack of BRMS1's effect on HIF activity made us question whether ING4 is associated with the mSin3A-HDAC1/2 complex which BRMS1 is a component of. HeLa cell nuclear extracts were immunoprecipitated with ING4 and BRMS1 immune sera and corresponding pre-immune sera and precipitated proteins were analyzed by western blotting to detect presence of HDAC2 protein (Fig. 3.3). HDAC2 was co-precipitated with both BRMS1 immune sera but not with ING4 immune sera or any of the pre-immune sera. This result indicates that ING4 interacts with BRMS1 that is not associated with HDAC2; however, it is possible that ING4 might specifically interact with HDAC1 bound BRMS1. Nevertheless, the functional consequences of ING4-BRMS1 interaction remain unclear.

Identification of ING4 interacting proteins: Biochemical purification of ING4 complex

As an alternative to yeast two-hybrid screen for identification of ING4 interacting proteins, we also set up to purify ING4 complex using biochemical techniques including column chromatography, ammonium sulfate precipitation, and immuno-affinity purification. HeLa cells were fractionated into cytoplasmic and nuclear protein extracts as the first step of purification and nuclear extract was used in later steps to eliminate potential non-specific interactions with cytoplasmic proteins. Lack of any predictable enzymatic activity of ING4, yet its ability to repress activity of some transcription factors and activate some others,

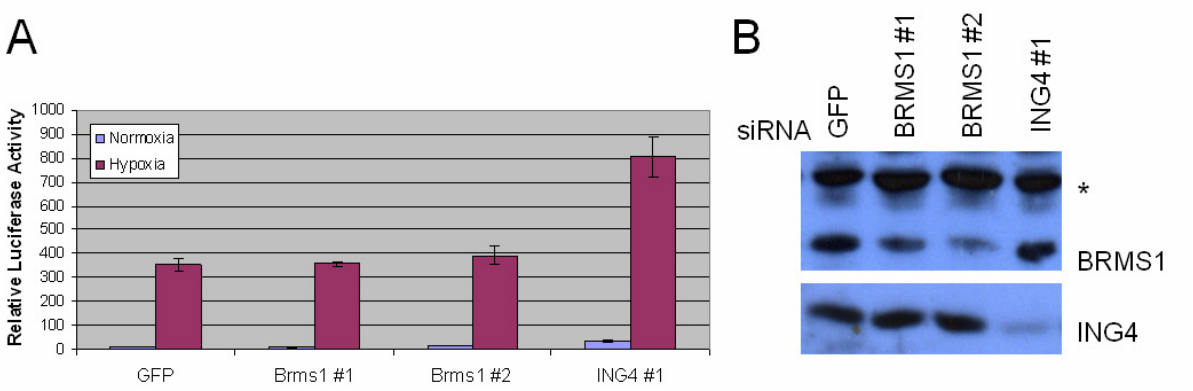


Figure 3.2 Suppression of BRMS1 has no effect on HIF activity.
(A) HeLa 3XHRE-tk-Luciferase stable reporter cell line transfected with GFP, BRMS1 (#1 and #2), and ING4 specific siRNA oligo. Luciferase activity of transfected cells was measured 3 days after transfection where cells kept under normoxic conditions or exposed hypoxic conditions for last 15 h.
(B) Proteins levels of BRMS1 and ING4 were detected by western blot analysis using specific antibodies. A non-specific band on BRMS1 blot indicating equal sample loading is marked by asterisks.

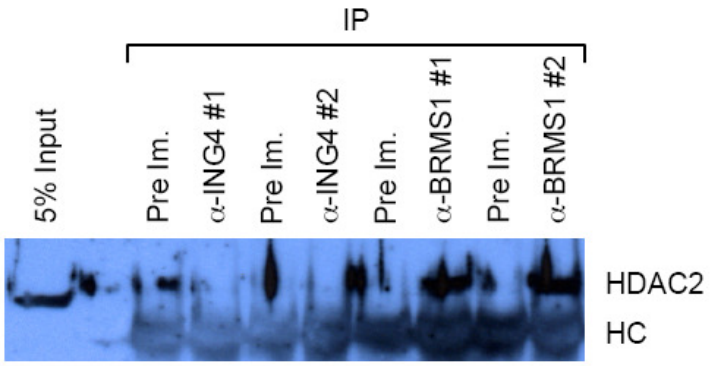


Figure 3.3 Brms1 but not ING4 interacts with HDAC2.
Interaction of BRMS1 and ING4 proteins with HDAC2 protein was tested by co-immunoprecipitation of HDAC2 from HeLa nuclear extract (0.4 M KCl) using ING4 and BRMS1 specific antibodies. Corresponding pre immune serum was used as a negative control. HC, heavy chain.

suggested that ING4 would reside in multiple protein complexes or a protein complex with multiple components. ING4 fractionated in high molecular weight fractions (>300 kDa) when HeLa nuclear extract was resolved on a size exclusion column (Fig. 3.4A) supportive of such ING4 complex.

Ammonium sulfate salt was added to nuclear extracts up to indicated percent saturations and proteins were separated into soluble (S: supernatant) and precipitated (P: pellet) protein fractions by centrifugation. Analysis of each fraction by western blotting revealed that ING4 was completely soluble up 24% ammonium sulfate saturation and completely precipitated by 40% (Fig. 3.4B).

Recombinant ING4 protein aggregated in buffer with pH lower than 6.95 (the calculated pI of ING4) with extended incubation while it remained soluble in buffer with higher pH (~8.0). Therefore, design of a successful purification scheme necessitated the sensitivity of endogenous ING4 to pH to be established. To this end, nuclear extracts were dialyzed against buffer with different pH and separated into soluble and precipitated protein fractions by centrifugation. Western blot analysis of each fractions indicated that similar to recombinant protein endogenous ING4 aggregated where the pH of buffer is lower than 7.0 (Fig. 3.4C). This limited the use of cation exchange columns in which efficient protein binding requires pH of the buffer to be at least 1 unit below the pI of the protein.

Binding and elution profile of ING4 present in HeLa nuclear extract was established using Q, DEAE, ANX, and SP HiTrap columns, and hydroxyapatite CHT II columns. Western blotting of HeLa nuclear extract fractions from HiTrap Q (Fig. 3.4D), HiTrap DEAE (Fig. 3.4E), and HiTrap ANX (Fig. 3.4F) anion-exchange columns ran with pH 8.0 buffers

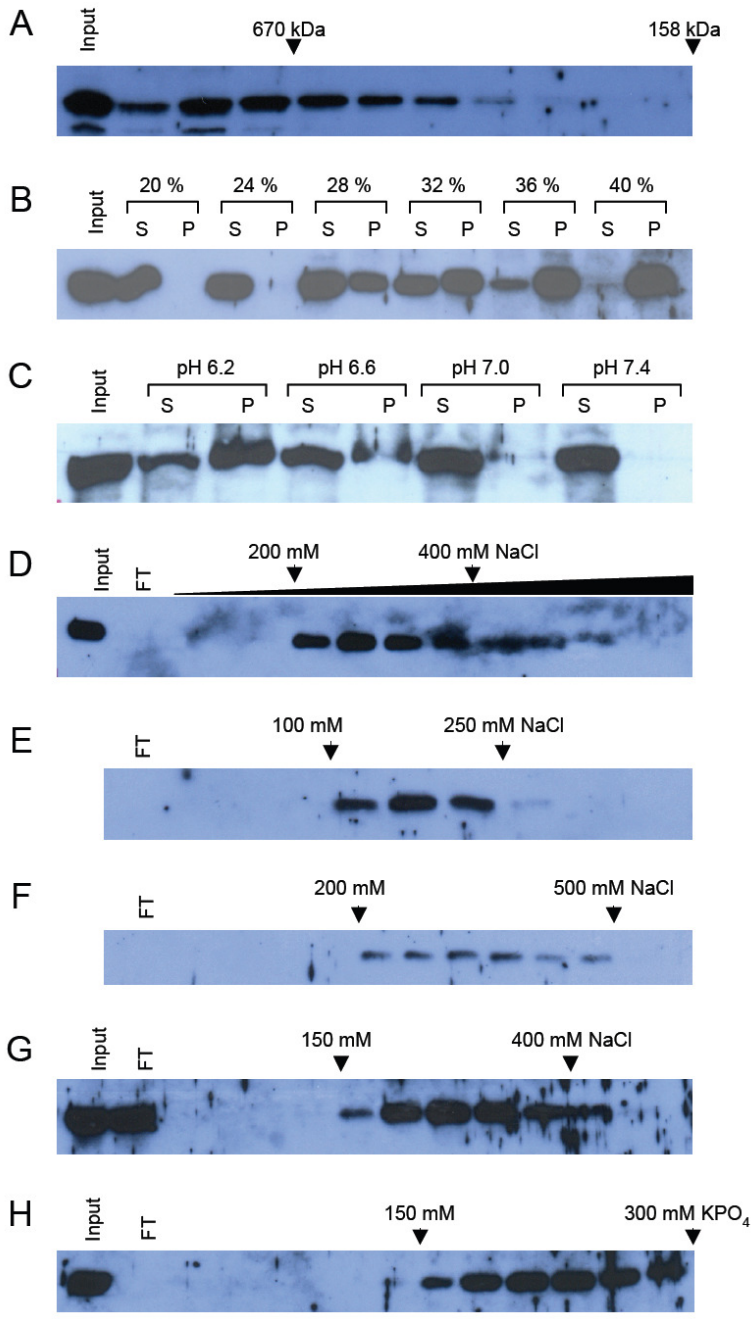


Figure 3.4 Characterization of ING4 for biochemical purification.
Proteins in HeLa nuclear extract were separated on Superdex 200 size exclusion column (A), precipitated with ammonium sulfate at indicated concentrations (B), dialyzed against different pH buffers and centrifuged to separate soluble (S) and precipitated (P) proteins (C), fractionated using anion exchange columns HiTrap Q (D), HiTrap DEAE (E), and HiTrap ANX (F), cation exchange column HiTrap SP (G), and BioRad CHT II ceramic hydroxyapatite column (H). Proteins in each fraction were analyzed by western blotting using ING4 specific antibody. Elution profile of protein size markers for size exclusion column and concentration of salt used for protein elutions are indicated at the top of each panel.

indicated that ING4 elutes between salt concentrations of 200-400 mM, 100-250 mM, and 200-500 mM NaCl, respectively. ING4 was efficiently bound to these columns under these conditions suggested by the absence of ING4 in flow through fractions (FT). ING4 eluted in between 150-400 mM NaCl concentration when nuclear extracts were ran on HiTrap SP column (Fig. 3.4G). For SP column fractionation a pH 6.6 buffer was used to prevent aggregation of ING4 (Fig. 3.4C). However, the close pH value of the buffer to that of ING4's pI value (~6.95) resulted in inefficient binding to column and loss of substantial amount of ING4 in the flow through fraction. ING4 was efficiently bound to CHT II hydroxyapatite column and eluted by 150-300 mM $K.PO_4$ when nuclear extract was ran over the column at pH 8.0 (Fig. 3.4H). Hydrophobic interaction columns could not be used for purification of ING4 since ING4 was not bound to Butyl, Octyl, and Phenyl HiTrap columns at as high as 1.5 M ammonium sulfate concentration (data not shown).

Based on the behavior of ING4 over different columns and methods of purification, we designed the scheme outlined in Fig. 3.5A to attempt to purify ING4 together with associated proteins. Starting with a 10 liter culture pellet of HeLa cells, nuclear proteins extract was prepared and proteins precipitated between 24-40% ammonium sulfate saturation were fractionated over a HiTrap Q anion exchange column. Q fractions of 200-400 mM NaCl were combined and applied to HiTrap DEAE column from which the 100-250 mM NaCl fractions were combined and fractionated with a CHT II hydroxyapatite column. 150-300 mM $K.PO_4$ fractions were combined and separated on HiTrap SP column at pH 6.6. SP column 200-400 mM NaCl fractions containing ING4 were pooled, dialyzed against buffer containing 200 mM $K.PO_4$ pH 6.6, 10 mM KCl, 5 mM β-mercaptoethanol, 0.3% NP-40, and

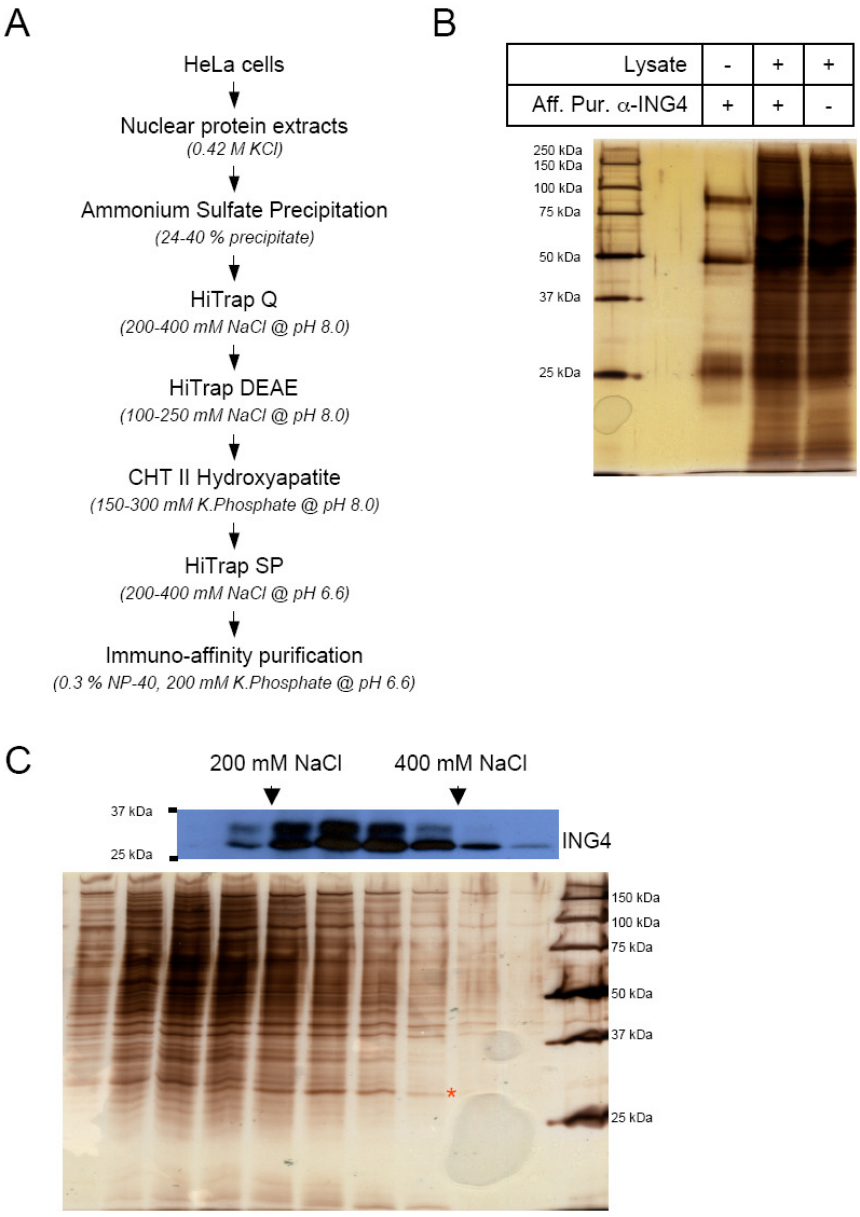


Figure 3.5 Partial purification of ING4 from HeLa cells.
(A) Summary of steps designed to purify ING4 starting with HeLa cells. Each method or column is listed in the order that they were used. Fractions containing ING4 that were combined and used in the next step are indicated in parenthesis.
(B) ING4 containing fractions from HiTrap SP column were combined and precipitated with Protein G-agarose resin in the presence or absence of affinity purified ING4 antibody. Precipitated proteins were resolved by SDS PAGE and silver stained.
(C) Fractions of HiTrap SP cation exchange column were separated by SDS PAGE and used for western blotting (top panel) or silver staining (bottom panel). A protein band with a similar size and elution profile to that of ING4 is indicated by red asterisks. Molecular weight of each size marker is indicated next to the protein band.

precipitated with Protein G-agarose beads in the presence and absence of affinity purified ING4 immune serum. Precipitated proteins were resolved by SDS PAGE and silver stained (Fig. 3.5B). Non-specific binding of proteins under these conditions, which were optimized for efficient immuno-precipitation of ING4, to Protein G-agarose beads prevented the detection of specifically precipitated proteins, if there were any. However, silver staining of proteins in SP column fractions revealed a protein band which resembles ING4 with regards to its size on gel (~30 kDa) and elution profile (Fig. 3.5C), suggesting a partial purification of ING4. Although this result was encouraging, identification of co-purifying proteins was impossible because of the excess contaminating proteins.

ING4 and HPH-2 do not co-purify in biochemical purification techniques

Having established the interaction between ING4 and HPH-2 in Chapter 1, we tested whether these two proteins might be co-purified. Consistent with Figure 3.4B, ING4 was soluble at 20% saturation of ammonium sulfate and completely precipitated at 40% saturation (Fig. 3.6A). In contrast, HPH-2 was soluble at 30% saturation and complete precipitation was observed at 50%. Even at 40% saturation HPH-2 was equally distributed between pellet and supernatant fractions whereas ING4 was already precipitated. Likewise, sensitivity of these proteins to pH differs from one another. Most of the ING4 protein aggregated at pH 6.2 while HPH-2 aggregated very little (Fig. 3.6B). Column elution profiles of ING4 and HPH-2 differ as well, most pronounced with CHT II hydroxyapatite column where almost a complete separation of the two proteins was observed (Fig. 3.6C). Taken together, these data suggest that ING4 and HPH-2 cannot be co-purified with one another

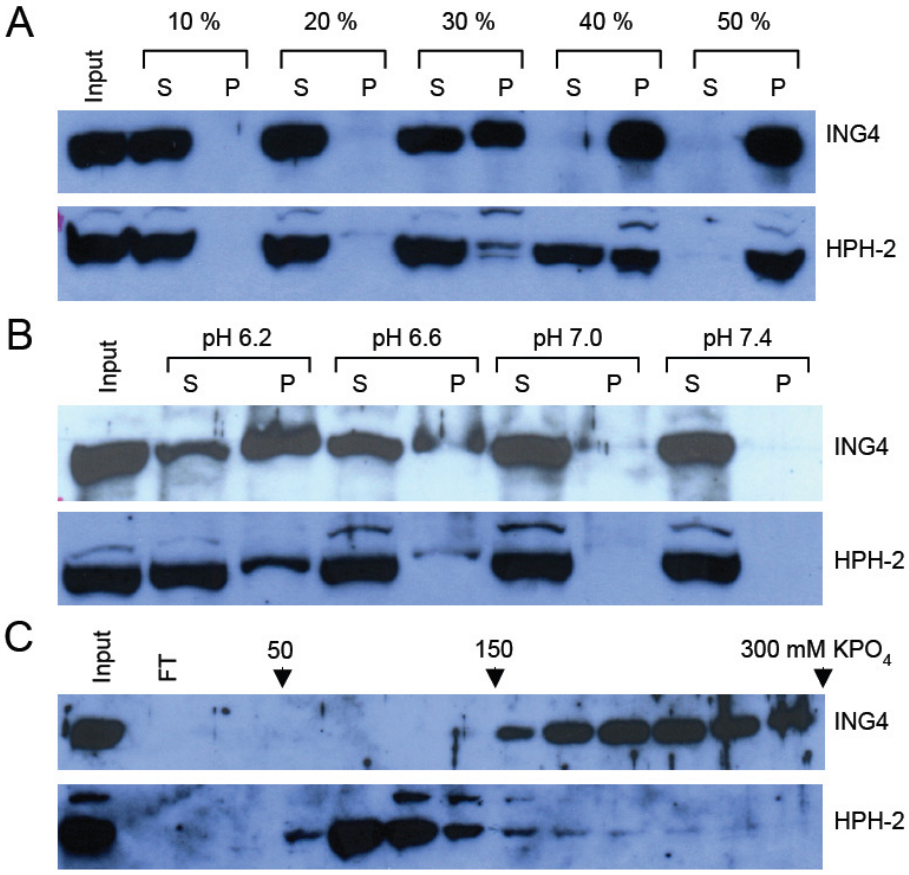


Figure 3.6 HPH-2 does not co-purify with ING4 in biochemical purification.
Proteins from HeLa nuclear extract were fractionated into precipitant (P) and supernatant (S) with ammonium sulfate at indicated concentrations (A) or with dialysis against different pH buffers (B). (C) Hydroxyapatite column bound HeLa nuclear proteins were eluted with a gradient of K.PO4. Proteins in each fraction were analyzed by western blot using ING4 and HPH-2 specific antibodies.

under conditions used for biochemical purification of ING4. Non-stoichiometric and/or weak interaction between the two proteins might contribute to the separation of ING4 and HPH-2 into different fractions in the aforementioned biochemical purification techniques.

Identification of ING4 interacting proteins: Tandem affinity purification of ING4

Our third alternative approach for identification of ING4 interacting proteins was tandem affinity purification (TAP) method. TAP method has been successfully used by J. Cote's group for purification of NuA4 histone acetyltransferases complex from human cells, which led to identification of ING3 as a subunit of this complex (Doyon, Selleck et al. 2004). Similar to one used in this work, an N-terminal tag cassette consisting of two Protein A domains-TEV protease cleavage site-Calmodulin binding peptide (CBP)-3XFLAG peptide (NTAP-3XFLAG) was generated and fused to ING4 (NTAP-3XFLAG-ING4). In order to avoid potential problems associated with transient transfection and overexpression, we attempted to generate HeLa stable cell lines with inducible expression of NTAP-3XFLAG-ING4. However, for unknown reasons it was impossible to get any NTAP-3XFLAG-ING4 expressing clones using Ponasterone A (Invitrogen), Tet-on, or Tet-off inducible systems (Clontech).

Transfection of HeLa cells with CMV-driven NTAP-3XFLAG-ING4 expression construct luckily yielded a stable clone in which the tagged ING4 was expressed at a level comparable to that of endogenous protein (Fig. 3.7A). Nuclear protein extracts prepared from this and an empty vector transfected cell lines were affinity purified first with IgG-sepharose resin and then with FLAG M2 agarose resin. Affinity purification with calmodulin resin was

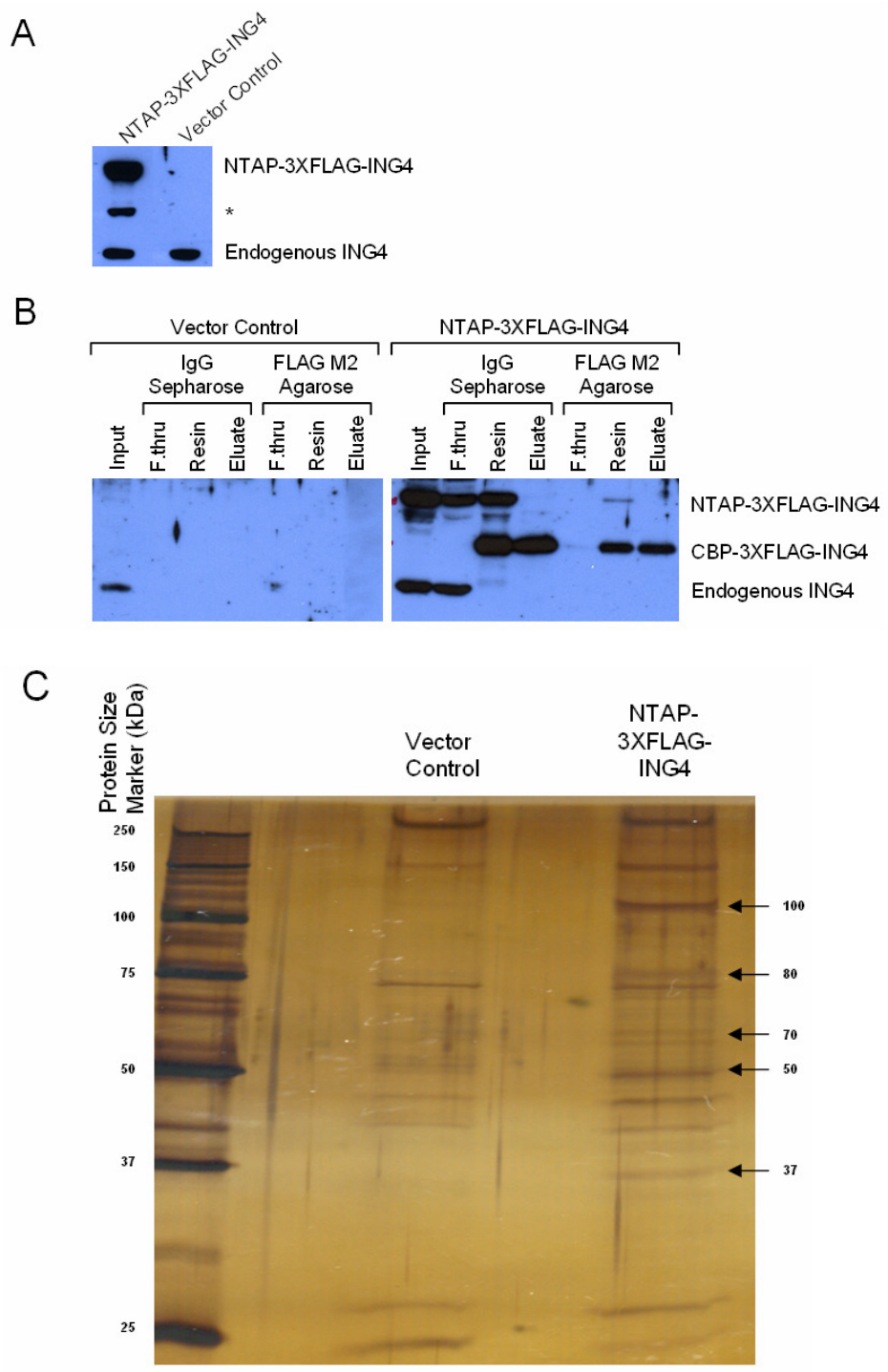


Figure 3.7 Tandem Affinity Purification of ING4.
(A) Expression level of NTAP-3XFLAG-ING4 and endogenous ING4 proteins in total cell lysates of HeLa cells stably transfected with NTAP-3XFLAG-ING4 expressing construct or empty vector were assayed with western blotting using ING4 immune serum. Degradation product of NTAP-3XFLAG-ING4 protein is indicated with an asterisk.

(B) Nuclear extracts of these stable cell lines were affinity purified sequentially with IgG-sepharose and FLAG M2-agarose resin. For both IgG and FLAG affinity purification step, proteins in flow through (F.thru), eluate, and those remain bound to resin after elution (resin) were analyzed by western blot using ING4 antibody.
(C) Final 3XFLAG peptide eluates of control and NTAP-3XFLAG-ING4 cell lines were resolved by SDS PAGE and silver stained. Specific ING4 co-purified protein band submitted for identification by mass spectrometry are indicated by estimated size of proteins. Molecular weight of protein size marker is shown on the left for reference.

skipped because of inefficient elution of bound proteins even with EDTA concentrations as high as 25 mM. Samples of starting nuclear extracts and flow through, post-elution resin bound proteins, and eluates from both affinity purification steps were monitored by western blot using α-FLAG M2 antibody (Fig. 3.7B). Endogenous ING4 protein was readily detectable in nuclear extracts of both cell lines and it did not co-purify with NTAP-3XFLAG-ING4 suggesting that either in contrast to the recombinant protein (Chapter 2), endogenous ING4 is monomeric or NTAP-3XFLAG-tag interferes with trimerization of ING4. Only about half of the NTAP-3XFLAG-ING4 protein present in nuclear extract was capture by IgG-sepharose resin and off of this half only ~40% was recovered by TEV digestion, the remaining ~60% was still bound to resin (~20% undigested and ~40% digested form). Eluted CBP-3XFLAG-ING4 protein was completely bound to FLAG M2 resin however, 50% of it was recovered by 3XFLAG peptide elution. The overall estimated efficieny of purification was around 10%. Final 3XFLAG peptide eluates of both cell lines were resolved by SDS PAGE and proteins were visualized by silver staining (Fig. 3.7C). Protein bands observed specifically in NTAP-3XFLAG-ING4 sample, indicated by estimated molecular weight on silver stained gel (Fig. 3.7C), were submitted to Protein Chemistry Core Facility for identification of proteins by mass spectrometry.

Results of mass spectrometric analysis of submitted protein bands are listed in Table IV. As expected, the 37 kDa band was identified as ING4 protein. Proteins in 50 and 70 kDa bands were not identifiable with certainty. The 80 kDa protein band was identified as MYST2/HBO1 protein. Fragmentation pattern of the 100 kDa band was consistent with two candidate proteins, hnRNP U and PHF16/JADE3, both of which are listed in Table 3.2.

Intuitively, one would have expected ING4 to bind a histone deacetylase based on it transcriptional repressor activity however, MYST2 is a member of the MYST family of histone acetyltransferases. Heterogenous nuclear ribonucleoprotein U (hnRNP U, also known as Scaffold Attachment Factor-A (SAF-A)) is a subunit of hnRNP complex with implicated functions in transcriptional regulation, RNA and protein trafficking, and signal transduction. JADE3/PHF16 is a PHD containing protein which has not been studied yet. However, JADE-1, a close homolog of JADE3 that is stabilized by pVHL, associates with histone acetyltransferases and acts as transcriptional coactivator.

BRMS1, an ING4 interacting protein identified in our yeast two hybrid screen, was not detectable in the final ING4 TAP eluate by Western blot analysis (data not shown), suggesting either a transient interaction between the two proteins or blockage of interaction because of the NTAP-tag fused to ING4.

ING4 interacts with MYST2 but not with hnRNP U

Interactions of ING4 with MYST2 and hnRNP U proteins were tested by co-immunoprecipitation experiments where transiently expressed 3XFLAG-tagged proteins were co-precipitated with endogenous ING4 using ING4 immune serum (Fig. 3.8A).

Table 3.2 Nano-HPLC/MS/MS identification of ING4 interacting proteins from HeLa stable cell line.

Gel Band ID	MW on Gel	Protein Name	Protein MW	GI Number
I-100	100	Heterogenous nuclear ribonucleoprotein U (hnRNP U)	87.9	32358
		PHD finger protein 16 (PHF16/JADE3)	93.4	7662006
I-80	80	MYST histone acetyltransferase 2 (MYST2/HBO1)	60.3	21619719
I-70	70	Protein unfound		
I-50	50	Protein unfound		
I-37	37	Inhibitor of growth family member 4 (ING4)	28.4	38201670

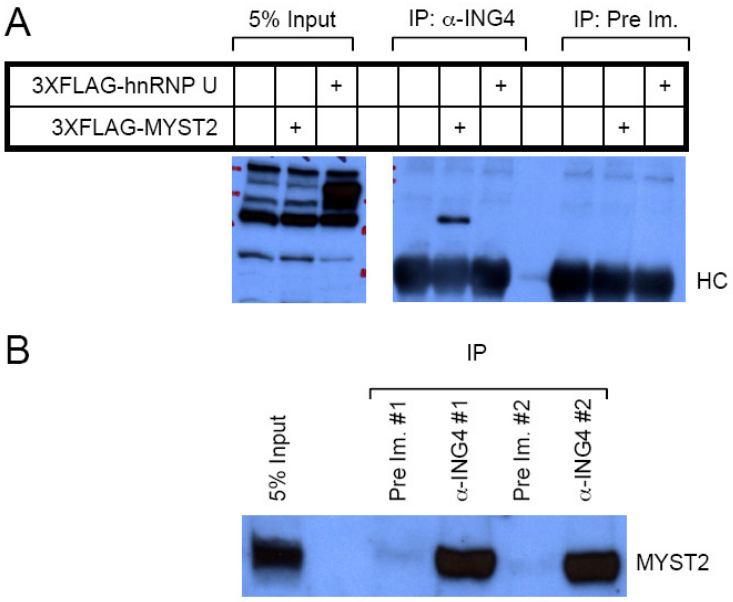


Figure 3.8 MYST2 but not hnRNP U co-immunoprecipitates with ING4.
(A) Transiently expressed 3XFLAG-tagged MYST2 and hnRNP U proteins were immunoprecipitated with ING4 antibody or pre immune serum. 3XFLAG-tagged MYST2 and hnRNP U proteins in 5 % of lysates used for immunoprecipitation and in precipitates were detected by western blotting using α-FLAG M2 antibody.
(B) MYST2 present in HeLa cell nuclear extract was immunoprecipitated with two different ING4 immune and pre-immune serums, and detected by western blotting with MYST2 specific antibody (MYST2 Ab#1). HC, Heavy chain.

Although overexpressed, 3XFLAG-hnRNP U did not co-precipitate with ING4 indicating that the 100 kDa band that co-purified with ING4 in tandem affinity purification might actually be the PHF16/JADE3 protein. 3XFLAG-MYST2 on the other hand, was efficiently precipitated with ING4 immune serum even though it was not detectable in input lysates due to a non-specific cross-reacting band. Pre-immune serum did not precipitate 3XFLAG-MYST2. Furthermore, MYST2 protein present in HeLa nuclear extract was precipitated with two different ING4 immune serums but not with the corresponding pre-immune serums (Fig. 3.8B). We focused our attention exclusively on MYST2 interaction of ING4 because of MYST2's histone acetyltransferase activity and prior literature implicating MYST2 in transcriptional regulation. By doing so, we have somewhat neglected the JADE3 interaction of ING4, which needs to be confirmed by alternative methods and studied further in future.

MYST2 binds to 3XHRE promoter but does not affect the reporter gene expression

Interaction of MYST2 with ING4 provided a potential explanation for the observed HIF repressor function of ING4 (Chapter 2). MYST2, when recruited as a component of ING4 complex by HPH-2, could inhibit transcriptional activity of HIF. Despite the general notion of histone acetyltransferases acting as transcriptional activators, transcriptional repressor effect of MYST2 histone acetyltransferase is not unprecedented. Although the underlying mechanism has not been elucidated, overexpression of MYST2 was shown to repress activity of androgen receptor (Sharma, Zarnegar et al. 2000). Similar repressive effect of MYST2 may provide an explanation for the activation of HIF in the absence of functional

ING4 protein. Lack of ING4 protein abolishes the recruitment of MYST2 protein thereby allowing HIF to become more active.

Chromatin immunoprecipitation with MYST2 antibody (α-MYST2 Ab#2) revealed that MYST2 interacted with 3XHRE promoter under hypoxic conditions (Fig. 3.9A). 3XHRE promoter was not immunoprecipitated by pre-immune serum (Pre Im.) or without any antibody (No Ab.), and consistent with data presented in Chapter 2, ING4 and ARNT immune serum were able to precipitate the 3XHRE promoter.

The functional relevance of MYST2 to hypoxia response pathway was studied by transient overexpression and by protein knock-down using siRNA oligos. Transient overexpression of MYST2, similar to ING4 (Chapter 2), did not affect luciferase expression in HeLa 3XHRE-tk-Luciferase stable cell line nor did it affect expression of endogenous HIF targets in HeLa measured by qRT-PCR (data not shown). Transfection of MYST2 specific siRNA oligos though yielded significant reduction in protein levels (Fig. 3.9B), did not affect the HIF-dependent luciferase reporter gene expression compared to GFP control siRNA (Fig. 3.9C). Likewise, suppression of MYST2 had no effect on HIF activity with shorter (5h) or longer (24h) incubation of cells under hypoxic conditions (Fig. 3.9D). Suppression of ING4 resulted in characteristic ~2 fold upregulation of luciferase reporter expression compared to GFP control following exposure of cells to hypoxia for 16 or 24 but not 5 hours.

ING4 and MYST2 repress NF-κB transcriptional activity

ING4 and MYST2 proteins, when transiently overexpressed, affected neither endogenous targets nor luciferase reporters of HIF and NF-κB transcription factors in HeLa

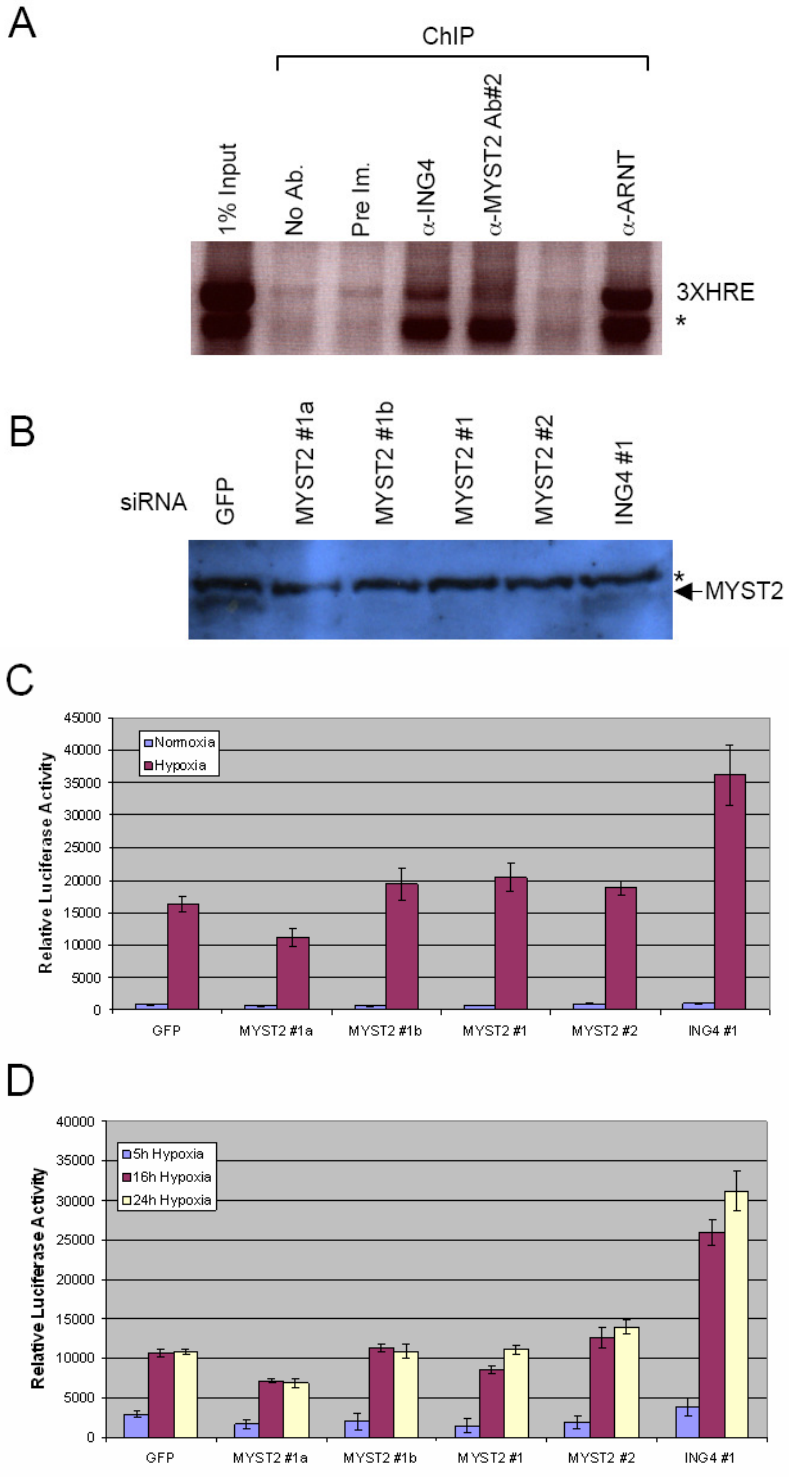


Figure 3.9 MYST2 binds to HIF target promoter but does not affect HIF activity.
(A) Chromatin fragments from hypoxia treated HeLa 3XHRE-tk-Luciferase cell line were immunoprecipitated with indicated antibodies/immune serums and pre-immune serum (Pre Im.) or without any antibody (No Ab.).

Precipitated DNA fragments were amplified by PCR. Smaller size PCR product in 3XHRE promoter amplifications is indicated with an asterisk.
(B) HeLa 3XHRE-tk-Luciferase stable reporter cell was line transfected with indicated siRNA oligos. MYST2 protein levels of each siRNA transfected cells were analyzed by western blot using MYST2 specific antibody. A non-specific band close to actual MYST2 protein band in size is marked with an asterisk.
(C, D) Luciferase activity of siRNA transfected cells was measured 3 days after transfection where cells kept under normoxic conditions or exposed to hypoxic conditions for the last 15 h (C) or exposed hypoxic conditions for 5, 16, or 24 h (D).

or U87MG cell lines (data not shown). In order to eliminate non-homogenous protein expression of transient transfections we generated stable cell lines overexpressing ING4 and MYST2. U87MG cells were used since it was previously shown that overexpression of ING4 repressed NF-κB targets IL6, IL8, and COX2 (Garkavtsev, Kozin et al. 2004). U87MG cells were transfected with either ING4 or 3XFLAG-MYST2 mammalian expression construct and two stable cell lines overexpressing each protein were selected for further study (Fig. 3.10A). N-terminal 3XFLAG-tagged MYST2 was used to avoid use of commercial MYST2 antibodies, none of which worked well in all three applications –western blotting, co-IP, and Ch-IP experiments– where MYST2 antibodies were used. Stable integration of ING4 expression construct into genomic DNA was verified by PCR amplification (Fig. 3.10A). ING4 and MYST2 mRNA levels, measured by quantitative real-time PCR (qRT-PCR), were significantly higher in both clones of each cell line than in empty vector transfected control cell line (Fig. 3.10B). In contrast to the control cell line, ING4 mRNA levels were slightly induced under hypoxic conditions in both ING4 transfected cell lines however, relevance of this to regulation of hypoxia response is not known.

Normoxic and hypoxic expression levels of HIF and NF-κB target genes were also analyzed by qRT-PCR (Fig. 3.10B). Expression of HIF target genes AK3 and NIP3 were not

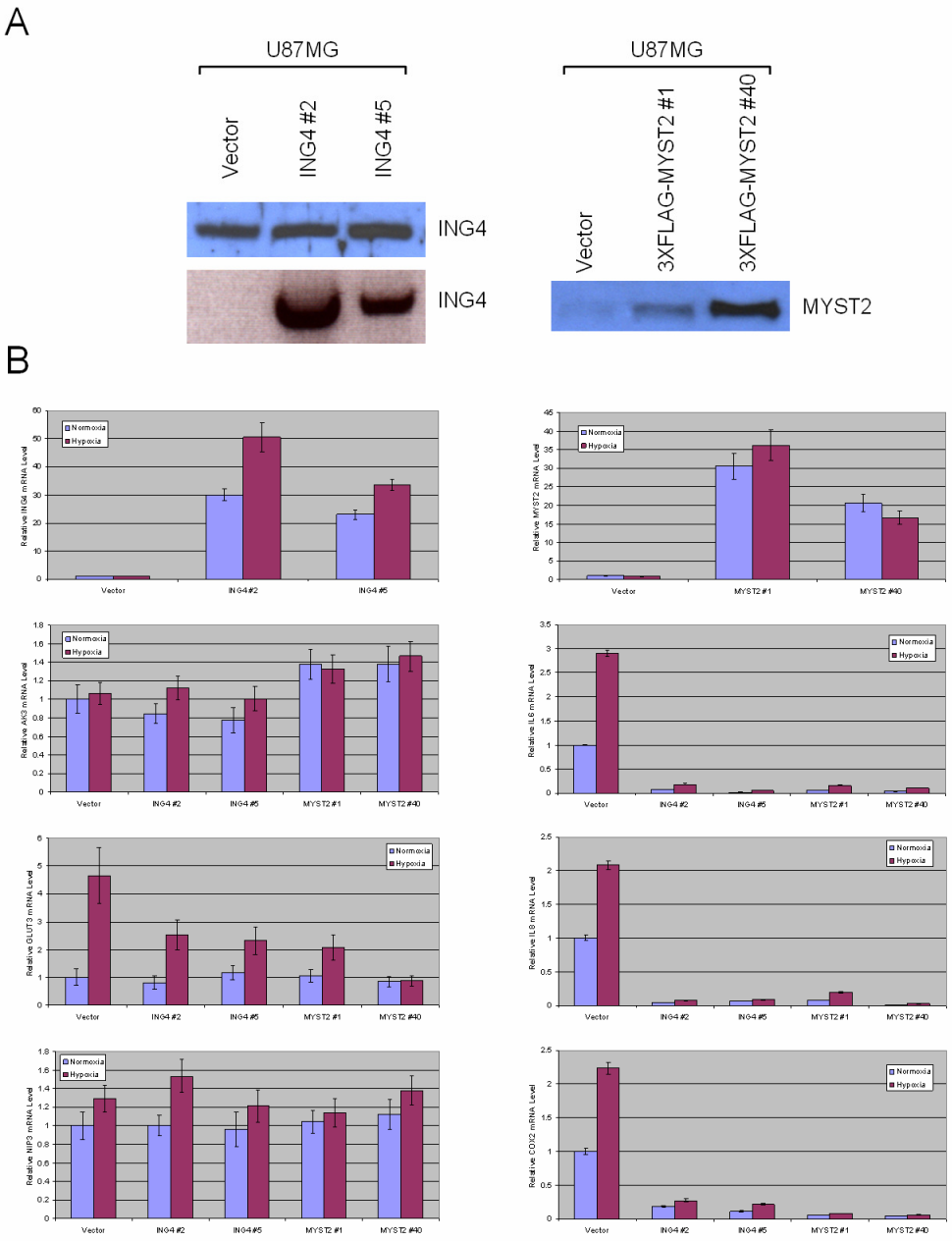


Figure 3.10 ING4 and MYST2 repress expression of NF-κB target genes in U87MG glioblastoma cell line. (A) U87MG cells were stably transfected with ING4 and 3XFLAG-MYST2 expression constructs or empty vectors. Expression level of each protein in lysates from equal number of cells was analyzed with western blot using ING4 (left top panel) and MYST2 specific antibodies (right panel). Stable integration of ING4 expression construct was confirmed by PCR amplification of ING4 from genomic DNA (left bottom panel). Primer pair contained one primer complementary to ING4 sequence and one to vector sequence.

(B) Expression of HIF and NF-κB target genes (AK3, GLUT3, NIP3, and IL6, IL8, COX2, respectively) were measured by quantitative real-time PCR (qRT-PCR) in each stable cell line grown under normoxic or hypoxic

conditions for 15 h. Expression level of each mRNA was normalized to cyclophilin mRNA in each sample and normoxic expression level in control cell line was assigned a value of 1 for comparison. Levels of ING4 and MYST2 were also measured to ensure overexpression of each construct.

induced by hypoxia and not affected by overexpression of either ING4 or MYST2. Expression of GLUT3, on the other hand, was induced by hypoxia and its hypoxic expression was reduced in ING4 and MYST2 overexpressing cell lines. All three NF-κB targets –IL6, IL8, and COX2– were induced under hypoxia. Both normoxic and hypoxic expression of these genes were dramatically repressed in ING4 and MYST2 stable cell lines suggesting ING4 and MYST2 proteins repress transcriptional activities of NF-κB and HIF.

ING4 and MYST2 interact with NF-κB

Having established repressive effects of ING4 and MYST2 on expression of NF-κB target genes we asked whether ING4 and MYST2 associate with NF-κB or their effects were transduced to NF-κB indirectly. Binding of ING4 and MYST2 to HIF target promoter (Fig. 3.9A) suggested that the mechanism of HIF regulation involves a direct protein-protein interactions between ING4/MYST2 and HIF. Although ING4 co-immunoprecipitated with NF-κB (Garkavtsev, Kozin et al. 2004), such an interaction has not been reported between MYST2 and NF-κB.

293 cells were transiently transfected with p65 NF-κB, 3XFLAG-ING4, and 3XFLAG-MYST2 expression constructs. Expressed ING4 and MYST2 proteins were precipitated with α-FLAG M2 antibody and co-precipitated p65 NF-κB protein was detected by western blotting (Fig. 3.11A). p65 NF-κB protein was not co-precipitated by α-FLAG M2

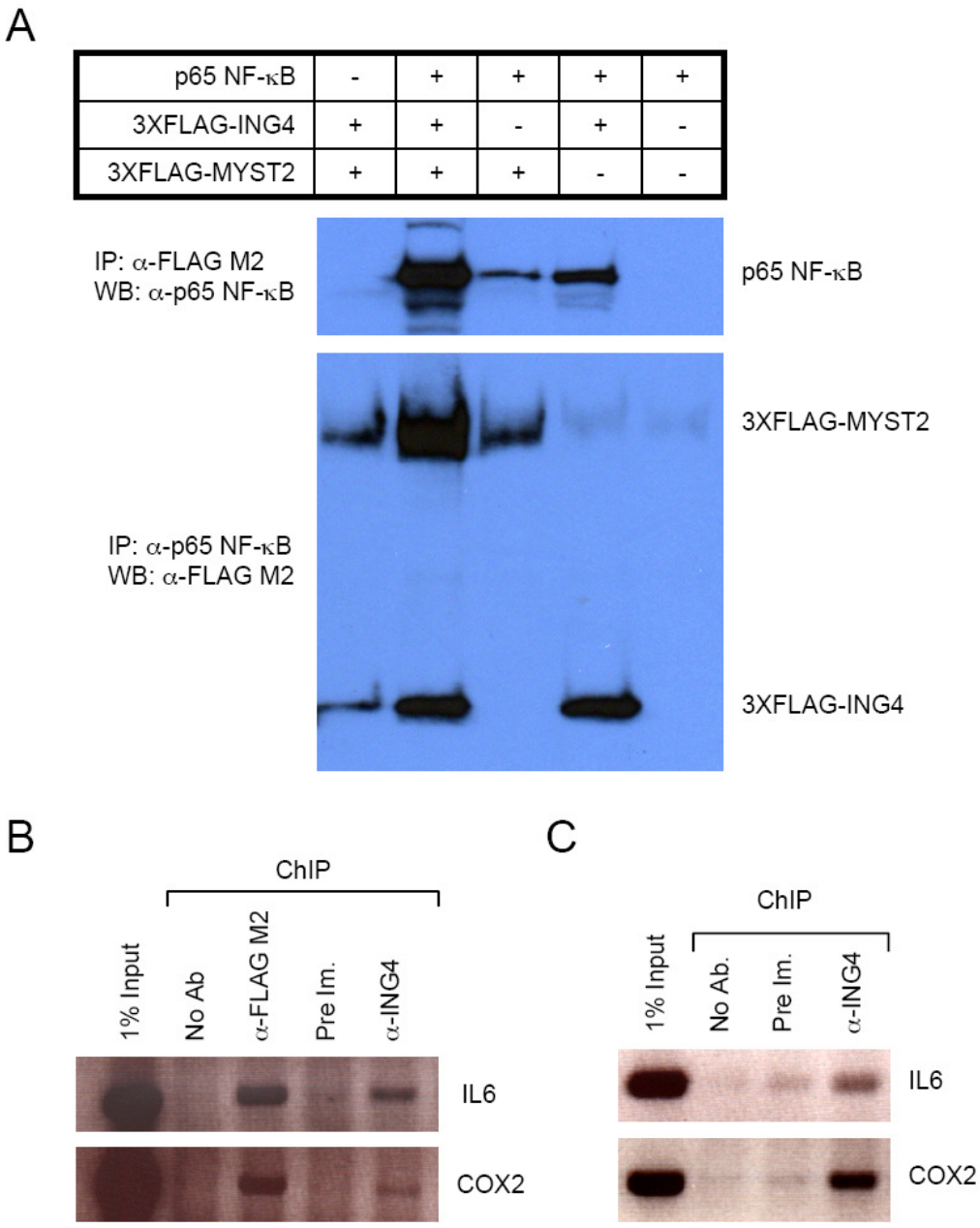


Figure 3.11 ING4 and MYST2 interact with NF-κB.
(A) Transiently expressed p65 NF-κB, 3XFLAG-ING4, and 3XFLAG-MYST2 proteins were immunoprecipitated with α-FLAG M2 antibody (top panel) or α-p65 NF-κB (bottom panel). Precipitated proteins were detected by western blotting using α-p65 NF-κB (top panel) and α-FLAG M2 antibody (bottom panel).
(B, C) Chromatin fragments from hypoxia treated U87MG 3XFLAG-MYST2 #1 (B) and HeLa (C) cells were immunoprecipitated with indicated antibodies/immune serums and pre-immune serum (Pre Im.) or without any antibody (No Ab.). Precipitated DNA fragments were amplified by PCR using IL6 and COX2 promoter specific primers.

antibody in the absence 3XFLAG-tagged ING4 and MYST2. However, it was co-precipitated when either 3XFLAG-ING4 or to a lesser extend when 3XFLAG-MYST2 were co-expressed. Precipitation of p65 NF-κB was greatly enhanced –more than additive– when 3XFLAG-ING4 and 3XFLAG-MYST2 were co-expressed and precipitated with α-FLAG M2 antibody. In a reciprocal experiment, α-p65 NF-κB antibody was used for immunoprecipitation and co-precipitated proteins were analyzed by western blotting using α-FLAG M2 antibody.

Chromatin immunoprecipitation experiments confirmed the binding of ING4 and MYST2 proteins to promoter regions of NF-κB target genes; IL6 and COX2 (Fig. 3.11B and 3.11C). Both IL6 and COX2 promoter regions were precipitated with ING4 immune serum from hypoxia treated U87MG MYST2 #1 stable cell line and HeLa cells, but not with pre-immune serum or in the absence of antibody (No Ab.). Likewise, α-FLAG M2 antibody was able to precipitate both IL6 and COX2 promoters. All together, these data indicate that ING4 and MYST2 bind NF-κB to repress its transcriptional activity.

Discussion

The candidate tumor suppressor protein ING4 has been implicated in multiple biological processes including transcriptional regulation (Shiseki, Nagashima et al. 2003; Garkavtsev, Kozin et al. 2004; Ozer, Wu et al. 2005), cell/tumor growth (Shiseki, Nagashima et al. 2003; Garkavtsev, Kozin et al. 2004), angiogenesis (Garkavtsev, Kozin et al. 2004), cell cycle control (Zhang, Xu et al. 2004), apoptosis (Shiseki, Nagashima et al. 2003; Zhang, Xu et al. 2004), and contact inhibition (Kim, Chin et al. 2004). It is well established that ING4

mediates (some of) its effects by altering activities of several transcription factors including HIF (Ozer, Wu et al. 2005), NF-κB (Garkavtsev, Kozin et al. 2004), and p53 (Shiseki, Nagashima et al. 2003). However, exactly how a non-enzymatic protein with a single recognizable protein-protein interaction domain (PHD) regulates multiple transcription factors has remained elusive. Other than the transcription factors themselves and HPH-2 (Chapter 2), the only other protein previously shown to interact with ING4 was the p300 transcriptional co-activator (Shiseki, Nagashima et al. 2003). This interaction provides some explanation for how ING4 might activate p53, but it also contradicts with the observed HIF and NF-κB transcriptional repressor functions of ING4. Convinced that ING4 can not perform transcriptional repression alone, we took multiple approaches to identify ING4 interacting proteins trying to elucidate the mechanism.

Breast cancer metastasis suppressor 1 (BRMS1) was identified as an ING4 interacting protein in yeast two-hybrid experiment. Consistent with the transcriptional repressor function of ING4, BRMS1 was shown to be a subunit of mSin3A-HDAC1/2 complex (Meehan, Samant et al. 2004; Nikolaev, Papanikolaou et al. 2004). A stable interaction between the founding member of ING protein family (ING1) and mSin3A-HDAC1/2 complex (Skowyra, Zeremski et al. 2001; Kuzmichev, Zhang et al. 2002), which was later shown to contain BRMS1 (Nikolaev, Papanikolaou et al. 2004), has been reported. Similar to ING1, ING2 was affinity purified together with mSin3A-HDAC1/2 complex and it was also noted that when overexpressed other members of the ING protein family could also interact with the same complex in addition to their specific protein complexes –ING3, NuA4/TIP60; ING4, MYST2/JADE3; ING5, MYST2/JADE1/2/3 and MOZ/MORF complexes (Doyon, Cayrou et

al. 2006). ING1 was also co-purified with the mSin3A complex containing subunits of BRG1-SWI/SNF chromatin remodeling complex (Kuzmichev, Zhang et al. 2002). Interestingly, BRG1-SWI/SNF complex has been implicated in regulation of NF-κB (Holloway, Rao et al. 2003) and HIF transcription factors (Wang, Zhang et al. 2004). BRG1-SWI/SNF complex recruited to COX2 promoter by NF-κB increases the accessibility of the promoter region by altering the nucleosome structure –chromatin remodeling– thereby enhances expression of COX2 (Holloway, Rao et al. 2003). Overexpression of either ATPase subunits of the SWI/SNF complex (BRAHMA and BRAHMA Related Gene 1 (BRG1)) were found to enhance erythropoietin (EPO), a HIF target gene, expression in response to hypoxia (Wang, Zhang et al. 2004). Conversely, suppression of BRG-1 protein led to reduced expression of EPO under hypoxic conditions. Although the exact mechanism was not delineated, it was proposed that the effect of SWI/SNF complex on EPO expression is mediated through chromatin remodeling.

Transient overexpression and siRNA knock-down of BRMS1 protein did not affect HIF transcriptional activity (data not shown and Fig. 3.2) and NF-κB inhibitory effects of BRMS1 has been well documented by others (Cicek, Fukuyama et al. 2005; Liu, Smith et al. 2006; Samant, Clark et al. 2007). However, we were unable to detect any effect on expression of NF-κB targets IL6, IL8, and COX2, (data not shown). In contrast to positive interaction between exogenous ING4 and BRMS1 in yeast two-hybrid and co-immunoprecipitation experiments (Fig. 3.1), western blot analysis of ING4 tandem affinity purification eluates revealed that endogenous BRMS1 does not co-purify with ING4 (data not shown). However, we can not completely rule out the possibility of a transient regulatory

interaction between ING4 and BRMS1 *in vivo*. Interaction of ING4 with BRMS1 (Fig. 3.1) but not with HDAC2 (Fig. 3.3) would be consistent with a model whereby binding of ING4 to a BRMS1 containing mSin3A/BRG1-SWI/SNF chromatin remodeling complex inhibits its activity thus leads to transcriptional repression. Though it is reasonable, this model is only speculative and requires experimental support.

While unbiased biochemical purification of protein/protein complexes is a very powerful technique for identification of interacting proteins, we were unable to purify ING4 complex to homogeneity this way. Separation of endogenous ING4 protein into >300 kDa molecular weight fractions on a size exclusion column (Fig. 3.4A), while recombinant ING4 behaved as a ~100 kDa protein (Chapter 2), supported the idea that ING4 resides in a stable protein complex which includes other proteins. Absence of endogenous ING4 protein within this complex (Fig. 3.7A) suggests that there is a single ING4 protein within the complex, as apposed to the trimeric nature of recombinant protein (Chapter 2). Aggregation below pH 7.0 (Fig. 3.4C) and hydrophilic nature of ING4 limited the use of cation exchange and hydrophobic interaction columns, respectively. With these limitations, it was only possible to partially purify ING4 while the co-purifying proteins could not be resolved (Fig. 3.5C).

Using a tandem affinity purification approach, we identified four proteins that co-purified with ING4 (Fig. 3.7). Mass spectrometric analysis of the protein bands yielded identities of only two of them (Table 3.2). The 80 kDa protein was identified as MYST2 histone acetyltransferase and analysis of the 100 kDa band was consistent with two candidate proteins namely JADE3/PHD zinc finger 16 (PHF16) and heterogenous nuclear ribonucleoprotein U (hnRNP U). Subsequent experiments confirmed the interaction between

MYST2 and ING4 (Fig. 3.8), and lack of detectable interaction with hnRNP U (Fig. 3.8A), suggested that the 100 kDa protein band was actually of JADE3 protein. Recently, Doyon *et. al.* also showed that ING4 forms a complex with MYST2 and JADE1/2/3, using the tandem affinity purification method (Doyon, Selleck et al. 2004). This further supports the identity of the 100 kDa protein in ING4 TAP eluate as JADE3. Furthermore, based on their results with the ING5-BRPF-MOZ complex in which JADE-like protein BRPF enhanced the binding of ING5 to MOZ, Doyon and coworkers have suggested that JADE1/2/3 proteins may mediate ING4-MYST2 interaction in a similar fashion.

Although MYST2 and ING4 associate with the 3XHRE promoter (Fig. 3.9A), suppression of MYST2 does not affect HIF-dependent luciferase reporter expression while suppression of ING4 induces it ~2-fold under hypoxic condition (Fig. 3.9C and 3.9D). Hypoxic but not normoxic expression of GLUT3, a HIF target gene, was repressed by stable overexpression of ING4 and MYST2 (Fig. 3.10B). AK3 and NIP3 genes were not even induced by hypoxia suggesting that they may not be regulated by HIF in U87MG cell line, thus they are not affected by overexpression of either ING4 or MYST2. However, expression of all three NF-κB targets (i.e. IL6, IL8, and COX2) that were analyzed were repressed by ING4 and MYST2 overexpressions. HIF, which might contribute to induction these genes under hypoxia, is likely to be repressed by ING4 and MYST2 lowering the expression of IL6, IL8, and COX2. There are at least three possible explanations of why MYST2 suppression might have not affected HIF and NF-κB transcriptional activity. First is the potential redundancy between the members of MYST histone acetyltransferase family (MOF/MYST1, HBO1/MYST2, MOZ/MYST3, and MORF/MYST4). ING5, the closest of

ING family proteins to ING4, interacts equally with MYST2, MOZ/MYST3 and MORF/MYST4 (Doyon, Cayrou et al. 2006). In the event of MYST2 elimination, either MOZ/MYST3 or MORF/MYST4 could replace MYST2 allowing ING4 complex to resume its transcriptional repressor function. Second possibility is the inefficiency of MYST2 knock-down. MYST2 antibody used for western blot analysis detected a weak protein band that was specifically knocked-down by transfection of MYST2 siRNA oligos (Fig. 3.9B). MYST2 protein might still be present in sufficient quantity but not detected by the antibody. Thirdly, the stronger protein band recognized by the α-MYST2 antibody could actually be a stabilized form MYST2 protein (Fig. 3.9B). This siRNA insensitive form of MYST2 might allow the ING4 complex to function properly.

ING4 is characterized as a candidate tumor suppressor protein since reduced expression levels (Garkavtsev, Kozin et al. 2004; Gunduz, Nagatsuka et al. 2005) and mutations of ING4 gene (Kim, Chin et al. 2004; Gunduz, Nagatsuka et al. 2005) have been observed in cancer cells. Recently, multiple splice variants of ING4 gene have been cloned (Unoki, Shen et al. 2006; Raho, Miranda et al. 2007). Full length ING4 is located exclusively in nucleus while shorter isoforms lacking or containing partial nuclear localization signals are distributed between cytoplasm and nucleus suggesting that functions of ING4 may not be restricted within nucleus. One aspect of ING4 biology that is how ING4 itself is regulated has never been investigated. It remains unknown whether expression and regulatory interactions of individual ING4 splice variants are dynamic/regulated or static/not regulated? If they are regulated, what is the stimulus that governs their expression and interactions with

other proteins? Are they regulated through a signaling pathway or can ING4 proteins function as direct sensors?

The PHD zinc finger is a candidate region for such sensory function. ING2, the first nuclear protein capable of binding phosphoinositides, selectively associates with a subset of these signaling molecules through its PHD zinc finger (Gozani, Karuman et al. 2003). Similarly, ING4 could potentially recognize such signaling molecules and initiate a transcriptional response. PHD zinc fingers recognize not only signaling molecules but also posttranslational modifications of proteins (Mellor 2006; Zhang 2006). Recently, PHD of ING4 was also shown to recognize histones 3 trimethylated at lysine 4 (Palacios, Garcia et al. 2006). Therefore, recruitment of ING4 complex could possibly be regulated by the methylation status of histones in chromatin structure. It is tempting to speculate that some of ING4's interactions might be mediated by methylation of the interaction partner as well.

CHAPTER FOUR
Identification of HPH and FIH-1 interacting proteins: Regulatory proteins or novel substrates?

Introduction

The main "switches" that regulate the Hypoxia Inducible Factor (HIF) transcription factor are the HIF prolyl and asparaginyl hydroxylases. HPH enzymes control the stability of the HIF α-subunit while FIH-1 alters the transcriptional activity of HIF. Since their identification, the biochemical characteristics, cellular localization, and regulation of HPHs and FIH-1 have been extensively studied. It was not until very recently, however, that the specific protein-protein interaction partners and novel substrates of these enzymes have been identified.

Proteins that are known to associate with HPH enzymes include substrate proteins, HIF-α subunits (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001; Ivan, Haberberger et al. 2002), and regulatory proteins; SIAH1/2 (Nakayama, Frew et al. 2004), TRiC complex (Masson, Appelhoff et al. 2004), and OS-9 (Baek, Mahon et al. 2005), and MORG1 (Hopfer, Hopfer et al. 2006). Previously identified FIH-1 interacting proteins include substrates: HIF-1α (Mahon, Hirota et al. 2001), p105 (NFKB1), and IκBα (Cockman, Lancaster et al. 2006), and regulatory proteins: SIAH1 (Fukuba, Yamashita et al. 2007), pVHL (Mahon, Hirota et al. 2001), and HDAC1/3 (Mahon, Hirota et al. 2001). What follows is a brief summary of these various interacting proteins.

Seven in Absentia Homolog 1 (SIAH1) and 2 (SIAH2) proteins are RING-finger domain containing E3-ubiquitin ligases, which are involved in the selective ubiquitination and degradation of multiple proteins (Matsuzawa, Li et al. 2003). Further studies investigating additional SIAH ubiquitination substrates yielded HPH proteins among others (Nakayama, Frew et al. 2004), as SIAH2 protein was shown to induce proteasomal degradation of HPH-1 and HPH-3 proteins. In the case for HPH-2, however, SIAH2 does not affect HPH-2 protein level even though it is able to bind HPH2. Furthermore, SIAH1 also targets FIH-1 to ubiquitin-mediated proteosomal degradation (Fukuba, Yamashita et al. 2007). In the overall setting of a hypoxic response, SIAH1 and SIAH2 proteins are induced leading to degradation of FIH-1 (Fukuba, Yamashita et al. 2007), HPH-1, and HPH-3 (Nakayama, Frew et al. 2004) thereby reducing HIF degradation and thus contributing to hypoxic stabilization and transcriptional activation of HIF α-subunits.

TCP-1 Ring Complex (TRiC, also known as Chaperonin Containing TCP-1 (CCT)) is a multi-subunit cytosolic chaperonin complex that is required for the folding of half a dozen proteins including the HIF-α E3 ubiquitin ligase pVHL (Dunn, Melville et al. 2001). Subsequent work showed that the TRiC chaperonin complex mediates assembly of the pVHL/Elongin B/Elongin C E3 ubiquitin ligase complex (Feldman, Thulasiraman et al. 1999). In addition, subunits of the TRiC complex were co-immunoprecipitated with HPH-1, suggesting that HPH-1 might be a novel substrate. Taken together, these results suggest a potential link between TRiC chaperonin activity and regulation of HIF response.

OS-9, initially identified as a HIF-1α interacting protein, was shown to interact with HPH-1 and HPH-2. When transiently overexpressed, OS-9 was found to enhance the

hydroxylation and degradation of HIF-1α (Baek, Mahon et al. 2005). However, it is not clear yet whether OS-9 directly affects the activity of HPH enzymes or it enhances hydroxylation by simply tethering the HIF-1α substrate to the HPH-2 enzyme.

MAPK Organizer 1 (MORG1), a WD-repeat protein, was identified as a HPH-3 interacting protein in a yeast two-hybrid screen (Hopfer, Hopfer et al. 2006). Similar to OS-9, overexpressed MORG1 represses HIF activity but interaction of MORG1 is likely to be restricted to HPH-3 since it was found to interact with HPH-3 through a unique N-terminal region not seen in other HPH enzymes. It is unknown how MORG1 functionally affects HIF or HPH-3.

In addition to SIAH1, FIH-1 was also found to interact with pVHL and HDAC1/3 (Mahon, Hirota et al. 2001). Prior to the discovery of its asparaginyl hydroxylase activity, FIH-1 was known to inhibit HIF transcriptional activity. Because it was suspected that FIH-1 might repress HIF transcriptional activity by recruiting histone deacetylases, interactions between FIH-1 and HDAC1/2/3 were tested and HDAC1 and HDAC3 were found to bind FIH-1 specifically. In the same report, FIH-1 was also shown to bind pVHL causing a stronger interaction between pVHL and HIF-1α.

As already mentioned, HPH and FIH-1 enzymes are the key regulatory factors for HIF-α, though HIF-α is not an exclusive substrate for these enzymes. HPH enzymes hydroxylate two proline residues (Pro402 and Pro564 of human HIF-1α) in the Oxygen-dependent Degradation Domain (ODD), whereas FIH-1 hydroxylates an asparagine residue (Asn803) in the C-terminal Transactivation Domain (CTAD) of HIF-1α. Mutational analysis revealed that most of the residues surrounding the target proline (even the conserved residues

of the LXXLAP motif) (Huang, Zhao et al. 2002) and asparagine (Linke, Stojkoski et al. 2004) can be mutated without a detrimental effects on hydroxylation. The lack of a specific hydroxylation motif makes identification of novel substrates of these enzymes difficult based on primary sequence analysis. Nevertheless, recent data suggest that there are additional substrates of HPHs and FIH-1 enzymes. FIH-1 hydroxylates p105 (NF-κB1), and IκBα though the consequence of this hydroxylation remains unclear (Cockman, Lancaster et al. 2006). Furthermore, HPH enzymes were implicated in hydroxylation-dependent regulation of IκB Kinase-β (IKK-β) (Cummins, Berra et al. 2006), and RPB1 subunit of RNA polymerase II (Kuznetsova, Meller et al. 2003), all of which contain an LXXLAP motif, and Iron Regulatory Protein 2 (IRP2) (Wang, Chen et al. 2004).

In order to identify HPH-1, HPH-2, HPH-3, and FIH-1 associated proteins (regulatory and/or substrate proteins), we purified each enzyme together with bound proteins using a tandem affinity purification method. HPH-1 co-purified with TRiC chaperonin complex and FIH-1, consistent with its dimeric structure, co-purified with endogenous FIH-1. To our surprise, HPH-2 co-purified with multiple proteins most of which are nuclear proteins suggesting unidentified nuclear functions for HPH-2 aside from HIF regulation. We also showed that HPH-2 can be methylated by Protein Arginine Methyltransferase 1 (PRMT1) and inhibition of methylation results in enhanced HIF activity under hypoxic conditions.

Experimental Procedures

Cloning

Protein coding sequences of HPH-1 (GenBank accession no. NM_022073) HPH-2 (NM_022051), HPH-3 (NM_053046), FIH-1 (NM_053046), and others were amplified by PCR and cloned into appropriate restriction sites of bacterial and mammalian expression plasmids. pHIS-, pGST-, and pMBP-parallel(Sheffield, Garrard et al. 1999), and pET28A (Novagen) plasmids were used for bacterial expression of fusion proteins. For mammalian expression of V5- and 3XFLAG-tagged proteins, pcDNA3.1/V5-HisA (Invitrogen) and p3XFLAG-CMV-10 (Sigma-Aldrich) plasmids were used. HPH-2 encodes for 426 amino acid full-length protein, while HPH-2C encodes HPH-2 C-terminal prolyl hydroxylase domain (a.acids 181-426), and 1KbSM20 encodes for a HPH-2 isoform which lacks amino acids 76-178 of full-length HPH-2. To generate methyltransferase inactive mutants of PRMT1 and PRMT5, S-adenosyl-L-methionine binding site of PRMT1 (72-GSGTG-76) and PRMT5 (365-GAGRG-369) were mutated by PCR to encode for RSRLE and RARLE, respectively(Rho, Choi et al. 2001). To generate constitutive mammalian expression plasmid for N-terminal TAP- and 3XFLAG-tagged (NTAP-3XFLAG-) proteins; two tandem repeats of Protein A domain, TEV protease cleavage site, Calmodulin binding peptide, and 3XFLAG peptide encoding DNA fragments were cloned into pcDNA3.1/V5-HisA plasmid. Protein coding sequences of HPH-1, -2, -3, and FIH-1 were cloned in frame 3' of the 3XFLAG peptide encoding sequence (Fig. 4.1). Every construct has been confirmed by DNA sequencing.

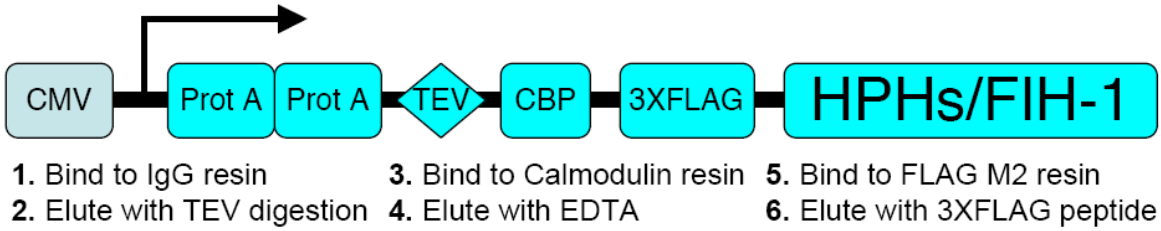


Figure 4.1 Tandem affinity purification of HPH-1, -2, -3, and FIH-1.
Mammalian expression constructs for N-terminal TAP-3XFLAG-tagged HPH-1, -2, -3, and FIH-1 proteins and proposed protocol for purification.

Recombinant protein expression and purification

Recombinant proteins were expressed in freshly transformed *E.coli* BL21(DE3)RIL strain (Stratagene). Expressions were induced with addition of IPTG to final concentration of 200 μM in LB media containing antibiotics (100 μg/ml ampicillin or 50 μg/ml kanamycin). Bacterial cultures were grown for 4-16 hours at 20-25 °C before storage of bacterial pellets at -80 °C.

6XHis-tagged proteins were purified with Ni-NTA agarose resin (Qiagen) in 25 mM Tris.Cl pH 8.0, 300 mM NaCl, 5 mM β-mercaptoethanol buffer and eluted with same buffer containing 250 mM imidazole. GST-tagged proteins were purified with Glutathione Sepharose 4B (GE Healthcare) resin in 1X PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4) buffer and eluted with 1XPBS containing 15 mM glutathione. MBP-tagged proteins were purified with Amylose resin (New England Biolabs) in 20 mM Tris.Cl pH 7.5, 200 mM NaCl, 5 mM β-mercaptoethanol buffer and eluted with same buffer containing 10 mM maltose. Homogeneity of recombinant protein samples was tested by Coomassie Blue staining following SDS PAGE. Protein concentration in each sample was estimated with Bradford assay (BioRad) using BSA solution as standard.

Antibodies and western blot analysis

Anti-serum against ING4, HPH-2, and FIH proteins were generated here at UT Southwestern animal facility against corresponding recombinant proteins. Two rabbits per protein (ING4: U5378 and U5379; HPH-2: U5429 and U5430; FIH: U5431 and U5432) were injected with Freud's adjuvant/recombinant protein mixture once every three weeks for four

times. 10 days after last injection animals were sacrificed and their blood were collected. Cleared-serum were aliquoted, stored at -80°C, and used for western blots at 1/2000 (U5378 and U5379) and 1/5000 (U5429, U5430, U5431, and U5432) dilutions. Commercially available antibodies against 3XFLAG- (Sigma) and V5-tags (Invitrogen) and HIF-1α (BD Biosciences), PRMT1, and PRMT5 (Abcam) proteins and dimethyl-arginine specific antibody Ab413 (Abcam) were purchased and used at manufacturer's recommended dilutions.

Proteins samples were separated on appropriate SDS polyacrylamide gel (8, 10, 12, or 15%) transferred to Hybond C-extra membrane. Membranes were blocked with 5% milk 1X PBST (1X PBS, 0.5% Tween-20) solution for 30min-1h, and incubated with primary antibodies diluted in 1% milk 1X PBST solution for 1-3h. Membranes were washed with 1X PBST solution for 10, 5, and 5 minutes prior to incubation with corresponding HRP-conjugated secondary antibodies (anti-rabbit, anti-mouse, or anti-goat) diluted 1/5000 in 1% milk 1X PBST solution for 30 min. Membranes were washed again with 1X PBST solution for 10, 5, and 5 minutes prior to detection with ECL-Reagent and exposure to X-ray film.

Cell culture and generation of stable cell lines

293 human embryonic kidney and HeLa human cervical cancer cell lines and stable cell lines derived from these parental cells were grown in DMEM High Glucose (HyClone) media supplemented with 10% Fetal Bovine Serum (HyClone), and antibiotics penicillin (100 U/ml) and streptomycin (0.1 mg/ml). 300 μg/ml of G418 was added to stable cell lines medium.

Stably transfected HeLa 3XHRE-tk-Luciferase and 293 Luciferase-HIF-2α ODD cells were used to monitor HIF transcriptional activity (luciferase expression controlled by HIF response elements (HREs)) and HIF-α subunit stability (stability of luciferase reporter controlled by HIF-2α ODD fusion), respectively. To monitor both simultaneously, 293 Nip3-Luciferase-HIF-1α ODD reporter cells, where expression was controlled by HIF-dependent Nip3-promoter and protein stability controlled by HIF-1α ODD fusion, were used.

To generate stable cell lines for tandem affinity purifications, HeLa and 293 cell transfected with NTAP-3XFLAG-protein expression constructs were selected in media containing 300 μg/ml G418 for two weeks. Clones tested positive for expression of the fusion protein underwent a second round of selection to get a better homogenous cell population. Clones with closest expression level to that of corresponding endogenous protein were selected and used for Tandem Affinity Purification (TAP).

Tandem affinity purification (TAP)

Stable cell lines expressing N-terminal TAP-tagged constructs were grown to 90-100% confluence under normoxic conditions. Cells from 50 15cm plates were used for final purification. Every step of purification was carried either on ice or in cold-room and centrifugation of resins were done at 1000g for 3 min. Cell pellets were resuspended in 5 packed cell volume TAP Lysis Buffer (TLB: 10% Glycerol, 50 mM HEPES-KOH pH 7.5, 100 mM KCl, 2 mM EDTA, 0.1% NP-40, 2 mM DTT, 0.25 mM $NaOVO_3$) and homogenized by passage through 22G1 needle. Lysates cleared by centrifugation (16000g, 30 min, 4 °C) were incubated with 200 μl IgG-sepharose resin (Amersham) pre-washed with TAP Lysis

Buffer. After 4 hours incubation on a rotater, resins were washed twice with TAP Lysis Buffer and trice with TEV Cleavage Bufer (TCB: 10 mM HEPES-KOH pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 0.1% NP-40, 1 mM DTT). Proteins were eluted from resin by 20-24h TEV digestion in 800 µl final volume containing 40 µg recombinant TEV. Following digestion, supernatants were collected and resin were washed twice with 400 µl modified Calmodulin Binding Buffer (mCBB: 10 mM HEPES-KOH pH 7.5, 200 mM NaCl, 1.5 mM Mg-Acetate, 0.1% NP-40, 1.5 mM imidazole, 15 mM β-mercaptoethanol, 3 mM $CaCl_2$), which were combined with TEV digestion supernatant. Calmodulin binding step was omitted in purification protocol due to inefficient recovery of proteins from resin even with up to 25 mM EDTA. Instead, TEV digestion eluates were incubated with 60 µl FLAG M2 agarose resin pre-washed with 1:1 mixture of TCB and mCBB. After 4h incubation on a rotater, resins were extensively washed with TCB:mCBB (1:1) buffer and proteins were eluted twice with 120 µl TCB:mCBB (1:1) buffer containing 400 ng/µl 3XFLAG peptide. Combined eluates were centrifuged again to remove residual resin and final eluates were separated on 10% SDS gel for silver staining(Shevchenko, Wilm et al. 1996). Specific protein bands were submitted to Protein Chemistry Core Facility (UT Southwestern Medical Center at Dallas) for identification by nano-HPLC/MS/MS.

For identification of HPH-2 and FIH-1 substrate proteins, a similar TAP was done in the presence of iron chealator desferioxamine (DFO). Stable cells grown for ~16h in media supplemented with or without 100 µM DFO (30 15cm plates per cell line per condition) were used for purifications. 100 µM DFO was supplemented in each step of purification and HPH

and FIH activity of final eluates were tested with ELISA- and GST pull-down-based assays, respectively.

siRNA transfections

Ready to use annealed siRNA oligos were purchased from Dharmacon (option A4), resuspended in supplemented 1X Universal Buffer at 100 μM concentration. 293 cell line stably transfected with HIF-2α ODD-Luciferase reporter construct was transfected twice with siRNA oligos at 100 nM concentration 24 hours apart with calcium phosphate transfection method. 60 hours post-transfection cells were either kept under normoxic or hypoxic condition for 15 hours. Lysates prepared from cells 75 hours post-transfection were used for measurement of luciferase activity and western blot detection of individual proteins. HeLa cells were plated in 24-well plates (3.0 10^4 cells/ml) with antibiotic-free DMEM High Glucose media supplemented with 10% FBS 16-20h prior to transfection. HeLa cells were transfected with 100 nM siRNA oligos using Oligofectamine Reagent (Invitrogen) following manufacturer's protocol. Transfected cells were assayed similar to 293 cells.

PRMT1 (also known as HRMT1L2) and PRMT5 (SKB1) duplex siRNA oligo sequences were as follows; PRMT1 #1, UCAAAGAUGUGGCCAUUAAUU and UUAAUGGCCACAUCUUUGAUU; PRMT1 #2, GCAACUCCAUGUUUCAUAAUU and UUAUGAAACAUGGAGUUGCUU; PRMT1 #3, GAUCGUGUGUUCCAGUAUCUU and GAUACUGGAACACACGAUCUU; PRMT1 #4, GCUACUGCCUCUUCUACGAUU and UCGUAGAAGAGGCAGUAGCUU; PRMT5 #1, CAACAGAGAUCCUAUGAUUUU and AAUCAUAGGAUCUCUGUUGUU; PRMT5 #3, UCAGACAUAUGAAGUGUUUUU and

AAACACUUCAUAUGUCUGAUU; PRMT5 #4, GAAGGGAUUUCCUGUUCUUUU and AAGAACAGGAAAUCCCUUCUU; PRMT5 #5, GCCCAUAACGGUACGUGAAUU and UUCACGUACCGUUAUGGGCUU.

Transient transfections: Calcium phosphate precipitation and lipofectamine reagent

293 cells were plated on 10 cm plates coated with Matrigel matrix (BD Bioscineces) with DMEM High Glucose, 10% FBS (No antibiotics) media so that cells are ~80% confluent at the time of transfection. 5-10 µg plasmid was mixed with 59 µl of 2M $CaCl_2$ in total volume of 500 µl. 500 µl of 2X HBS (8.0g NaCl, 0.37g KCl, 201mg Na_2HPO_4•7H2O, 1.0g dextrose, 5.0g HEPES/500ml (adjust pH to 7.05 with NaOH, filter sterilize, and store at 4°C)) was added drop-wise to DNA-$CaCl_2$ mix while vortexing. Final 1ml transfection mix was then added directly to cells in 10 cm plates. Cells are assayed 24-48h post-transfection.

HeLa cells were plated in 24-well plates (7.5 10^4 cells/ml) with DMEM High Glucose, 10% FBS (No antibiotics) media 16-20h prior to transfection. HeLa cells were transfected with 0.4 µg plasmid per well using Lipofectamine Reagent (Invitrogen) following manufacturer's protocol. Transfected cells were assayed 24-48h post-transfection.

Co-immunoprecipitation

293 cells were transfected with indicated expression constructs. 36-48h post-transfection cells were lysed with FLAG IP Lysis Buffer (20 mM Tris.HCl pH 7.5, 150 mM NaCl, 1% Triton X-100) freshly supplemented with protease inhibitor cocktail (Sigma-Aldrich). Lysates cleared by centrifugation (13000 rpm at 4°C for 15 min) were incubated

with FLAG-M2 agarose beads for 4 hours in cold-room on a shaker. Following extensive washes of beads with FLAG Wash Buffer (50 mM Tris.HCl pH 7.5, 150 mM NaCl), bound proteins were eluted with 2X SDS sample buffer (125 mM Tris.HCl pH 6.8, 4% SDS, 20% glycerol, 0.004% bromophenol blue) and separated on SDS polyacrylamide gels together with 2-10% input for subsequent western blotting.

HPH activity assay: ELISA

Non-transparent adhesive 96-well plates (Corning) were coated with neutravidin and then with biotinylated peptide substrates (Tuk-2539; HIF-1α a.acids 556-574) or HIF-1α ODD (a.acids 400-600) recombinant protein (N-terminal 6XHis- or GST-tagged). Peptide Tuk-2473 which is same as Tuk-2539 except Pro564 replaced with 4-hydroxyproline, was used as positive control. Excess non-bound substrate was washed with 1X PBS and hydroxylation reactions were started with the addition of recombinant enzyme (N-terminal 6XHis- or MBP-tagged 1KbSM20 or 6XHis-HPH2C) to equal volume 2X reaction buffer (40 mM Tris.HCl pH 7.5, 3 mM $MgCl_2$, 10 mM KCl, 4 mM DTT, 1 mM 2-oxoglutarate, 2 mM ascorbate, 20 μM Fe(II)). Hydroxylation reactions were carried out at room temperature under normoxia for 30 minutes in 100 μl total volume per well. Reactions were terminated by washing enzyme away from substrate using 1X PBS solution. Extend of substrate hydroxylation was then measured with hydroxyproline specific antibody (# 4817), HRP-conjugated anti-rabbit secondary antibody, and enhanced chemoluminescence (ECL) reagent. Light emitted from each well was measured with BioTek luminescence microplate reader.

To measure endogenous HPH activity, cells were washed twice with ice-cold 1X PBS on ice and lysed with three packed cell volume of hypotonic buffer HB (20 mM HEPES-KOH pH 7.5, 5 mM NaF, 10 μM Na_2MoO_4, 0.1 mM EDTA) supplemented with 0.5% NP-40. Cell debris was removed by centrifugation (13000 rpm, 15 min, 4 °C). Total protein concentration was determined by Bradford Assay (BioRad), and 100 μg protein was used for hydroxylation of peptide substrate in HPH ELISA assay.

[^{14}C]-2-oxoglutarate decarboxylation assay: Hydroxylase activity

Hydroxylation of candidate HPH substrate proteins was measured by a modified [^{14}C]-2-oxoglutarate decarboxylation assay derived from Ref(Kivirikko and Myllyla 1982). Briefly, a 1-ml reaction containing 3.0 μM peptide substrate (HIF-1α a.acids 556–574) or recombinant proteins were incubated with recombinant MBP-1KbSM20 enzyme in the presence of 50 mM Tris_HCl pH 8.0, 2 mg/ml BSA, 0.2 mg/ml catalase, 5 mM KCl, 1.5 mM MgCl2, 1.0 mM DTT, 64 μM [^{14}C]-2-oxoglutarate [14.6 nCi/nmol specific activity], 50 μM ascorbate, and 20 μM $FeSO_4$ in a sealed 15-ml tube. After one hour incubation at room temperature with gentle shaking, the pH of the solution was lowered to ~2 with diluted $HClO_4$. Released [^{14}C]-CO2 was captured by 3M Whatman paper saturated with 10 M NaOH and measured either by scintillation counting or exposed to PhosphorImager screen (Amersham), scanned by Typhoon Phosphorimager System and quantitated by ImageQuant software (Molecular Dynamics).

FIH-1 activity assay: GST pull-down based assay

Recombinant GST-p300 CH1 domain fusion protein was bound to glutathione resin in buffer containing 20 mM Tris.Cl pH 8.0, 100 mM NaCl, 10 μM $ZnCl_2$, 0.5% NP-40, 0.5 mM DTT, and Protease Inhibitor Cocktail. Radiolabeled HIF-2α C-terminal Trans-Activation Domain (CTAD; a.acids 774-874) was prepared with rabbit reticulocyte lysates in vitro transcription-translation system (Promega) in the presence of [^{35}S]-methionine. Radiolabeled substrate was hydroxylated with FIH-1 enzyme in the presence of 2 mM 2-oxoglutarate, 2 mM ascorbate and 62.5 μM $FeSO_4$ for 1 hour at 30 °C and incubated with GST-p300 CH1 domain bound resin for 1 hour at 4 °C. After extensive washes with buffer (20 mM Tris.Cl pH 8.0, 100 mM NaCl, 10 μM $ZnCl_2$, 0.1% NP-40, 0.5 mM DTT, 0.1 mM PMSF), associated [^{35}S]-HIF-2α CTAD was detected either by exposure to PhosphorImager screen following SDS PAGE or scintillation counting.

In vitro protein methylation: PRMT1 and PRMT5

Methylation of recombinant proteins was carried out at 30 °C (at 4 °C when hydroxylase activity of methylated protein will be assayed). 30 μg substrate protein was incubated with 10 μCi S-adenosyl-L-[^{14}C-methyl]-methionine (Perkin Elmer) and 10 μg MBP-PRMT bacterially expressed enzyme (or immunoprecipitate by FLAG M2 antibody from transfected 293 cells) in 25 μl total volume adjusted with PRMT Methylation Buffer (50 mM Tris.Cl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF). Reactions were terminated with addition of SDS sample buffer. Proteins were separated on 10% SDS polyacrylamide gels and stained with coomassie blue. Stained gels were scanned and dried on Whatman paper, which were then exposed to Phosphorimager screens

(Amersham), scanned by Typhoon Phosphorimager System (Molecular Dynamics) and quantitated by ImageQuant software (Molecular Dynamics). Non-radiolabeled SAM (Sigma Aldrich) was used instead for methylation reactions when methylated 1KbSM20 protein was submitted to Protein Chemistry Technology Center core facility for methylation site identification by mass spectrometry.

Methyltransferase inhibitor (AdOx) treatment

Adenosine-2',3'-dialdehyde (AdOx) was purchased from Sigma-Aldrich and resuspended in 25% DMSO, aliquoted and stored at -20°C. Media on stably transfected cell lines were replaced with media containing AdOx at indicated time points prior to luciferase assay. Cells were kept under normoxic conditions except for indicated amounts of time under hypoxic conditions.

Results

Identification of FIH-1 interacting proteins from HeLa: Tandem affinity purification

Transfections of HeLa cells with CMV-driven N-terminal TAP-3XFLAG-tagged HPH-1, HPH-2, HPH-3, and FIH-1 expression constructs yielded stable cell lines of NTAP-3XFLAG-FIH-1. For reasons that are not clear, only a few cell colonies had survived G418 selection following NTAP-3XFLAG-HPH transfections and none of them had any detectable expression of the fusion proteins. Total cell lysates of empty vector and NTAP-3XFLAG-FIH-1 expression construct transfected stable cell lines were used for tandem affinity purification. Mass spectrometric analysis of the protein bands purified specifically from

NTAP-3XFLAG-FIH-1 cells, indicated by the estimated molecular weights of proteins on silver stained gel (Fig. 4.2), identified the 50 kDa protein as an antibody heavy chain and multiple candidate proteins for the 40 kDa protein (Table 4.1). As we predicted based on the calculated molecular weight of CBP-3XFLAG-FIH-1 protein, the 45 kDa protein band was identified as FIH-1 protein. The 50, 45, and 40 kDa protein bands were undetectable (or at best barely detectable) in the final TAP eluates of empty vector transfected cells. A fourth protein band, indicated by an asterisk on the silver stained gel, was not reproducibly co-purified with CBP-3XFLAG-FIH-1 in multiple purification, therefore, it was not submitted for mass spectrometric identification. Consistent with the previously established homodimeric structure of FIH-1 (Dann, Bruick et al. 2002; Elkins, Hewitson et al. 2003; Lee, Kim et al. 2003), endogenous FIH-1 –a ~40 kDa protein– was detectable in NTAP-3XFLAG-FIH-1 eluate by western blot analysis using α-FIH-1 immune serum but not with α-FLAG M2 antibody while both efficiently recognized ~45 kDa CBP-3XFLAG-FIH-1 protein. Although FIH-1 was not one of the candidate proteins identified by mass spectrometry for the 40 kDa band, we believe that endogenous FIH-1 constitutes at least a portion of this protein band.

Squamous cell carcinoma antigen 1 (SCCA1), also known as SERPINB3, was the most likely candidate protein for 40 kDa protein based on the quality of the match between the detected and calculated mass of peptide fragments. SCCA1, a potent inhibitor of serine (or cysteine) proteinases, is thought to regulate physiological and pathophysiological events involving proteolytic processing such as protein turn-over and tumor progression (Silverman, Bartuski et al. 1998). With the exciting possibility that FIH-1 hydroxylase activity could be

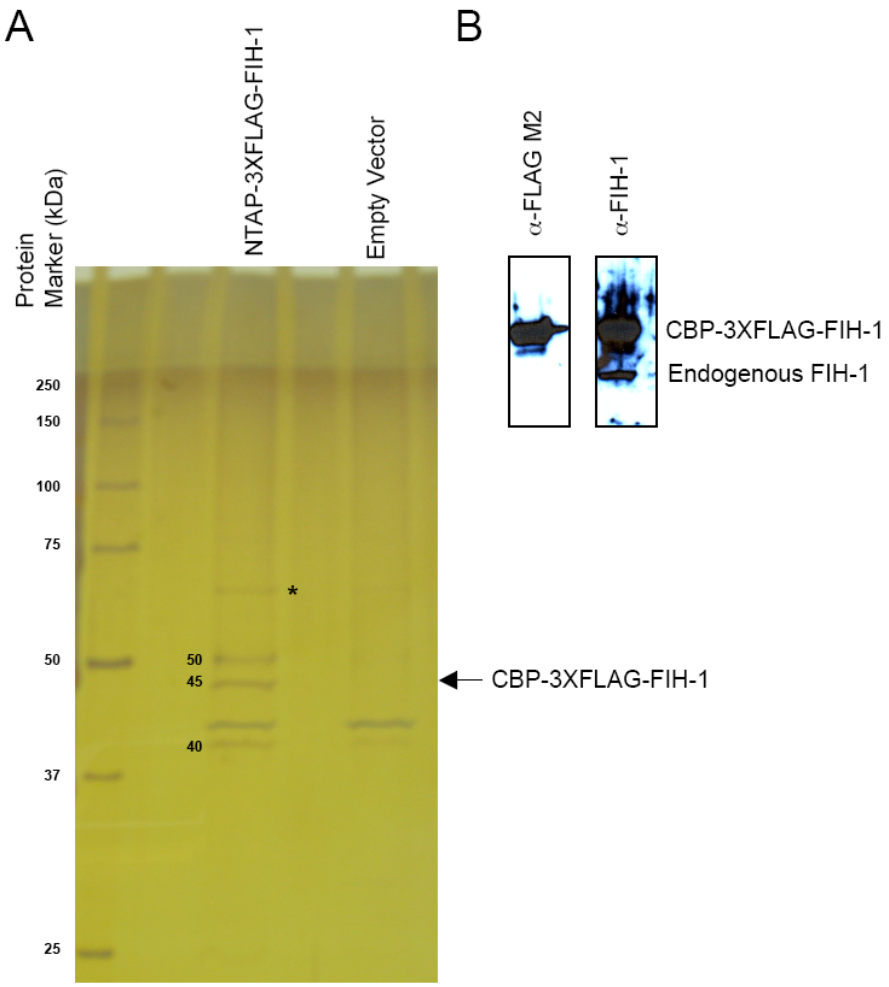


Figure 4.2 Tandem affinity purification of FIH-1 from HeLa cells.
(A) Affinity purified proteins of HeLa cells stably transfected with NTAP-3XFLAG-FIH-1 expression construct or empty vector were separated by SDS PAGE and silver stained. Protein bands specifically co-purified with NTAP-3XFLAG-FIH-1 (indicated by approximate molecular weight (kDa) on silver stained gel) were submitted to Protein Chemistry Core Facility for protein identification. A protein band (indicated by an asterisk) which was not reproducibly co-purified therefore it was not submitted for mass spectrometric identification.
(B) Western blot analysis of purified proteins with α-FLAG M2 and α-FIH-1 antibodies.

Table 4.1 Nano-HPLC/MS/MS identification of affinity purified FIH-1 interacting proteins from HeLa stable cell line.

Gel Band ID	MW on Gel	Protein Name	Protein MW	GI Number
F-50	50	Anti-colorectal carcinoma heavy chain	50.6	425518
F-45	45	Factor Inhibiting HIF 1 (FIH-1)	40.3	14043456
F-40	40	Serine (or cysteine) proteinase inhibitor, clade B member 3 (SERPINB3/SCCA1)	44.5	5902072
		SCCA1 protein isoform 1	44.6	33317676
		Beta actin	41.7	4501885
		Tubulin alpha 6	49.9	13436317
		Glyceraldehyde-3-phosphate dehydrogenase	36.0	31645
		Desmoglein 1 preproprotein	113.6	4503401
		Pyruvate kinase	57.8	35505
		Unknown (protein for IMAGE:3906970)	21.1	15928913
		Ubiquitous Mitochondrial Creatine Kinase	43.1	7767140
		Fructose bisphosphate aldolase	39.4	312137
		Annexin I	35.0	442631
		Hypothetical protein	40.3	34364597
		YWHAZ protein	35.3	30354619
		Methylosome protein 50 (MEP50)	36.7	13129110

regulated by SCCA1, we tested and did not detect an interaction between these two proteins with co-immunoprecipitation experiments (data not shown). The other candidate proteins for the 40 kDa band have not been followed upon primarily because of two reasons; either these proteins had low scores in mass spectrometric analysis or they are abundant proteins (i.e. actin, tubulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) and are common contaminants of affinity purifications.

Identification of HPH-1, HPH-2, HPH-3, and FIH-1 interacting proteins from 293 cells: Tandem affinity purification

After multiple unsuccessful attempts at generating HeLa stable cell lines with either inducible (tetracycline (Tet-on and Tet-off) and ponasterone A inducible expression systems) or constitutive (CMV promoter-driven) expression of NTAP-3XFLAG-tagged HPH proteins, we were finally able to establish 293 stable cell lines with CMV-driven mammalian expression constructs. Western blot analysis confirmed expression of NTAP-3XFLAG-tagged proteins and tags-alone (NTAP-3XFLAG-) in cell lysates (Fig. 4.3A, left panel). Each protein was affinity purified sequentially over IgG-sepharose and α-FLAG M2-agarose resins and eluted as CBP-3XFLAG-fusion proteins (Fig. 4.3A, middle panel). Similar to purification from HeLa cells (Fig. 4.2B), endogenous FIH-1 protein was co-purified with CBP-3XFLAG-FIH-1 from 293 cells (Fig. 4.3A, right panel). Co-purification of endogenous FIH-1 protein was a good indication for purification of native-like protein complexes under conditions used for the tandem affinity purification, since dimerization is required for FIH-1 hydroxylase activity (Dann, Bruick et al. 2002; Lancaster, McNeill et al. 2004).

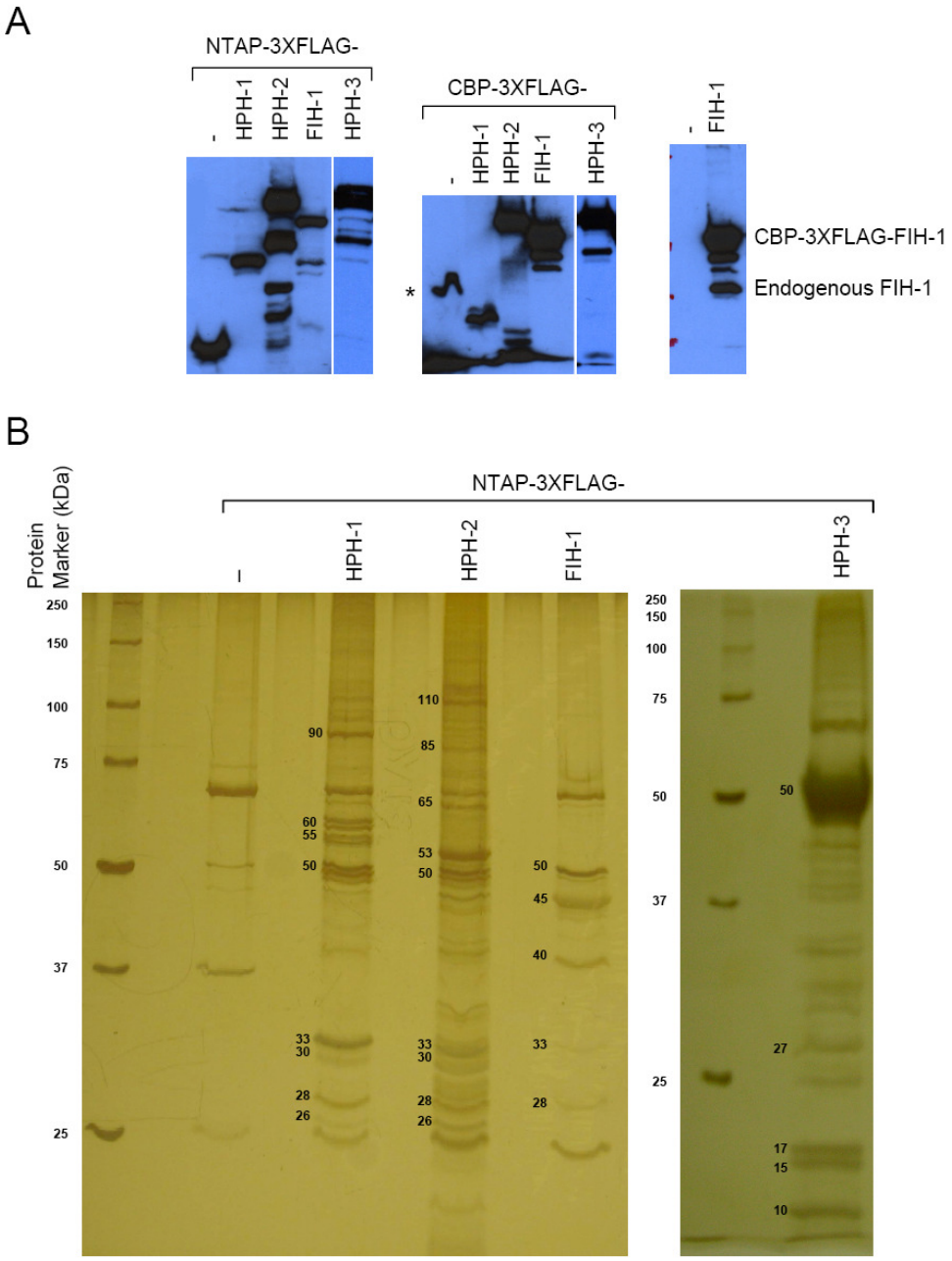


Figure 4.3 Tandem affinity purification of HPH-1, -2, -3, and FIH-1 from 293 cells.
(A) Lysates and final TAP eluates of 293 cells stably transfected with NTAP-3XFLAG-tagged HPH-1, -2, -3, and FIH-1 or tags-alone expressing constructs were separated by SDS PAGE and immunoblotted with α-FLAG M2 antibody (left and middle panels) and α-FIH-1 immune serum (right panel).
(B) Specifically co-purified protein bands that were submitted to Protein Chemistry Core Facility for identification are indicated by the approximate molecular weight (kDa) of each protein on silver stained gel. HPH-1 specific bands 1-60 and 1-55 were duplets, 1-50 and HPH-2 band 2-50 were triplets with equal intensity, therefore submitted as single sample for protein identification.

Proteins within final eluates were separated by SDS PAGE and silver stained (Fig. 4.3B). Purification starting with lysates derived from control cells expressing tags alone yielded four protein bands with a major non-specific protein band around 70 kDa that was also present in eluates of other proteins. Faint bands at 50 kDa and 25 kDa are likely to be heavy and light chains of α-FLAG M2 antibody, respectively. The size (37 kDa) and cross-reactivity with α-FLAG M2 antibody of the fourth protein band (Fig. 4.3A, middle panel indicated by asterisks) are consistent with it being NTAP-3XFLAG protein. However, elution of this protein from IgG-sepharose resin without TEV digestion is intriguing. The tag protein expected to be purified, CBP-3XFLAG (>10 kDa), ran out of the silver stained gel (Fig. 4.3B) but it was detected by western blot analysis at the dye front (Fig. 4.3A, middle panel). Based on the silver stained gel results, the HPH-1 eluate contained 12 protein bands that were not present in control purification while HPH-2 had 11, HPH-3 had 5, and FIH-1 had 5 specific bands. Protein bands in HPH-1 and HPH-2 eluates that were very close in size and intensity were submitted as a single sample (HPH-1: 1-60 doublet, 1-55 doublet, 1-50 triplet; HPH-2: 2-50 triplet) whereas the other distinct bands indicated by estimated molecular weight of each protein were submitted separately for protein identification. HPH-3 eluate contained one major protein band of 50 kDa while FIH-1 eluate contained three major specific protein bands which were consistent with proteins purified from HeLa (Fig. 4.2A) and two additional minor protein bands, 33 kDa and 28 kDa in size. Proteins that matched peptide fragments detected by mass spectrometry for HPHs and FIH-1 co-purified proteins are listed in Tables 4.2-4.5, for HPH-1, HPH-2, HPH-3, and FIH-1, respectively.

Table 4.2 Nano-HPLC/MS/MS identification of HPH-1 interacting proteins from 293 stable cell line.

Gel Band ID	MW on Gel	Protein Name	Protein MW	GI Number
1-90	90	Heat shock 90kDa protein 1, beta	83.2	15215418
		Heat shock protein HSP 90-alpha 4	84.6	61656605
		TCP1/ring complex (TriC) subunit 2 (CCT2)	57.4	48146259
		Alpha-tubulin	50.1	37492
		KIAA1228 protein	93.9	20521804
		Egl nine homolog 3 (EGLN3/HPH-1/PHD3)	27.2	11545787
1-60 (X2)	60	Hsp70/Hsp90-organizing protein (STIP1)	62.6	54696884
		TCP1/ring complex (TriC) subunit 5 (CCT5)	59.4	12804225
		TCP1/ring complex (TriC) subunit 3 (CCT3)	60.4	14124984
		TCP1/ring complex (TriC) subunit 6A (CCT6A)	57.7	62089036
		Chaperonin (HSP60)	61.0	306890
		Stimulator of TAR RNA binding	57.8	1200184
		Siah binding protein 1 (SIAHBP1)	58.0	1809248
1-55 (X2)	55	TCP1/ring complex (TriC) subunit 7 (CCT7)	59.3	56789228
		TCP1/ring complex (TriC) subunit 2 (CCT2)	57.4	48146259
		TCP1/ring complex (TriC) subunit 1 (CCT1)	60.3	57863257
		TCP1/ring complex (TriC) subunit 4 (CCT4)	57.9	76827901
		TCP1/ring complex (TriC) subunit 8 (CCT8)	58.5	1136741
		Alpha-tubulin	50.1	37492
		90kDa heat shock protein (HSP90)	83.2	306891
		Insulin receptor substrate 4	133.7	4504733
1-50 (X3)	50	Beta-tubulin	49.8	37494
		Anti-colorectal carcinoma heavy chain	50.6	425518
		hnRNP H1	49.1	48145673
		RuvB-like 2	51.1	12653319
1-33	33	Egl nine homolog 3 (EGLN3/HPH-1/PHD3)	27.2	11545787
		Similar to ribosomal protein S3a	30.0	14755682
		Unknown (protein for IMAGE:4110141)	27.6	14043538
		Emerin (Emery-Dreifuss muscular dystrophy)	29.0	57284204
		CGI-102 protein	24.0	4929673
1-30	30	Egl nine homolog 3 (EGLN3/HPH-1/PHD3)	27.2	11545787

		Ribosomal protein L7a	30.0	36647
		Semenogelin I (SEMGI)	45.3	32450803
		Semenogelin II precursor (SEMGII)	65.4	4506885
		Human pre-mRNA splicing factor SF2p32	30.9	338043
1-28	28	Lambda-chain precursor (a.a. -20 to 215)	24.6	33395
		Ribosomal protein L10a	24.8	531171
1-26	26	Chain C of the Antigen-Binding Domains From Three Variants Of Humanized Anti-P185	23.4	442925
		Unknown (protein for MGC:23888)	26.2	18490211
		Chain C of The Fab Fragment of a Human Monoclonal IgM Cold Agglutinin	23.3	10835794
		Ribosomal protein L14	23.8	1620022

Table 4.3 Nano-HPLC/MS/MS identification of HPH-2 interacting proteins from 293 stable cell line.

Gel Band ID	MW on Gel	Protein Name	Protein MW	GI Number	Co-IP
2-110	110	Nucleolin	74.3	21750187	–
		MOV10, Moloney leukemia virus 10 homolog	113.6	12803447	+
		MGC2641 protein	62.8	14495677	
		TRIM28 nuclear co-repressor protein	36.6	951332	+
		hnRNP U protein	88.9	32358	
2-85	85	DRBP76 alpha	60.5	9714266	–
		Nucleolin	74.3	21750187	–
		Ku80, ATP-dependent DNA helicase II	82.7	17512093	+
		DEAD-box polypeptide 50 (DDX50)	82.5	55664207	+
		HSP70, BiP protein	70.9	6470150	
2-65	65	Replication protein A1 (RPA1)	68.1	46430939	+
		Skb1 methyltransferase (PRMT5)	72.7	2323410	+
		IGF2 mRNA binding protein 1 (IGF2BP1)	63.4	7141072	+
		RNA-binding region (RNP1, RRM) containing 2	59.3	10635692	
		DEAD-box polypeptide 5 (DDX5)	66.9	226021	–
		hnRNP Q2 (NSAP1)	58.7	21619168	
		Nucleolin	76.3	128841	–
2-55	55	Egl nine homolog 1 (EGLN1/HPH-2/PHD2)	46.0	55665459	

2-50 (X3)	50	Alpha-tubulin	50.1	37492	
		Egl nine homolog 1 (EGLN1/HPH-2/PHD2)	46.0	55665459	
		Anti-colorectal carcinoma heavy chain	50.6	425518	
		Beta-tubulin	49.8	37494	
		FKBP8	48.6	31874548	+
		hnRNP H1	49.1	48145673	
		RuvB-like 2	51.1	12653319	
		Retinoblastoma binding protein 7	47.8	4506439	
		Glutamate-rich WD repeat containing 1	49.4	12803253	
2-33	33	Ribosomal protein S3a	30.0	45219787	
		Hornerin precursor	282.2	40795897	
		hnRNP A1	38.8	133254	
		Egl nine homolog 1 (EGLN1/HPH-2/PHD2)	46.0	55665459	
		Ribosomal protein S6	28.7	337516	
		Similar to ribosomal protein S2	31.4	27501336	
		CGI-102 protein	24.0	4929673	
		Transcriptional co-activator ALY (THOC4)	24.5	2896146	
		Emerin (Emery-Dreifuss muscular dystrophy)	29.0	57284204	
		HRS	21.5	2435501	
2-30	30	Ribosomal protein L7a	30.0	36647	
		Human pre-mRNA splicing factor SF2p32	30.9	338043	
		Replication protein A2 (RPA2)	29.2	18204222	+
		Transcriptional co-activator ALY (THOC4)	26.9	30411083	
		Ribosomal protein L8	28.0	15341853	
2-28	28	Ribosomal protein L10a	24.8	531171	
		Similar to ribosomal protein S8	24.2	55644275	
		Egl nine homolog 1 (EGLN1/HPH-2/PHD2)	46.0	55665459	
2-26	26	Ribosomal protein L14	23.8	1620022	
		Chain C of the Antigen-Binding Domains from Variants of Humanized Anti-P185	23.4	442925	
		Ig kappa chain NIG26 precursor	23.5	7438711	
		Chain C of the Fab Fragment of a Human Monoclonal IgM Cold Agglutinin	23.3	10835794	
		Egl nine homolog 1 (EGLN1/HPH-2/PHD2)	46.0	55665459	

Interactions of HPH-2 that have been confirmed by co-immunoprecipitation (Co-IP) are indicated by + sign, interactions that failed are indicated by – sign, and the ones that have not been tested are left blank.

Table 4.4 Nano-HPLC/MS/MS identification of HPH-3 interacting proteins from 293 stable cell line.

Gel Band ID	MW on Gel	Protein Name	Protein MW	GI Number
3-50	50	Egl nine homolog 2 (EGLN2/HPH-3/PHD1)	43.6	16604260
		Unknown (protein for IMAGE:4110141)	27.6	14043538
3-27	27	Egl nine homolog 2 (EGLN2/HPH-3/PHD1)	43.6	18031805
		Similar to ribosomal protein S8	24.2	55644275
		Ribosomal protein L10a	24.8	531171
		Hornerin precursor	282.2	40795897
		Unknown (protein for IMAGE:4110141)	27.6	14043538
		Chain L of the Fab Fragment of the Monoclonal Antibody Mak33	23.4	10835838
3-17	17	Egl nine homolog 2 (EGLN2/HPH-3/PHD1)	43.6	16604260
		Unknown (protein for IMAGE:4110141)	27.6	14043538
		Ribosomal protein L18	21.6	18204442
3-15	15	Egl nine homolog 2 (EGLN2/HPH-3/PHD1)	43.6	16604260
		Unknown (protein for IMAGE:4110141)	27.6	14043538
		Ribosomal protein L18	21.6	18204442
		PR310 c-K-ras oncogene	16.9	180592
3-10	10	Egl nine homolog 2 (EGLN2/HPH-3/PHD1)	43.6	16604260
		v-Ha-ras Harvey rat sarcoma viral oncogene homolog	21.3	54695712
		Unknown (protein for IMAGE:4110141)	27.6	14043538
		Ribosomal protein L21	17.6	619788
		Chain A of Calmodulin N-Terminal Domain	8.5	16974825

Table 4.5 Nano-HPLC/MS/MS identification of FIH-1 interacting proteins from 293 stable cell line.

Gel Band ID	MW on Gel	Protein Name	Protein MW	GI Number
F-50	50	Anti-colorectal carcinoma heavy chain	50.6	425518
		Tubulin alpha 6	49.9	13543589
		Human Serum Albumin	66.0	4389275
F-45	45	Factor Inhibiting HIF 1 (FIH-1)	40.3	14043456
		Unknown (protein for IMAGE:4110141)	27.6	14043538
F-40	40	Factor Inhibiting HIF 1 (FIH-1)	40.3	14043456
		Methylosome protein 50 (MEP50)	36.7	13559060
F-33	33	Hypothetical protein	30.7	8655689
F-28	28	Anti-colorectal carcinoma heavy chain	50.6	425518
		Hypothetical protein	30.7	8655689

Furthermore, we speculated that inhibition of HIF prolyl and asparaginyl hydroxylases may stabilize their interaction with substrates, thus, we also attempted to identify novel substrates of these enzymes by tandem affinity purification. To this end, HPH-2 and FIH-1 enzymes were affinity purified in the presence and absence of an iron chelator compound, desferrioxamine (DFO), which has long been used to deplete Fe(II) co-factor required for the activities of HPH and FIH-1 enzymes (Dann and Bruick 2005; Clifton, McDonough et al. 2006; Ozer and Bruick 2007). Neither expression (Fig. 4.4A, top panel) nor purification (Fig. 4.4A, middle and bottom panels) of HPH-2 and FIH-1 fusion proteins was affected by 100 μM DFO. Likewise, dimerization of FIH-1 remained unaffected as well (Fig. 4.4A, bottom panel). HPH-2 purified in the absence of DFO was able to hydroxylate HIF-1α peptide substrate as measured by an ELISA based assay, while HPH-2 purified in the presence of DFO had no HPH activity (Fig. 4.4B). FIH-1 eluates, regardless of the DFO status, had no HPH activity. Similarly, FIH-1 purified in the absence of DFO was active as assessed by asparaginyl hydroxylation-dependent blockage of interaction between the HIF-1α CTAD and the p300 CH1 domain in a GST pull-down assay format (Fig. 4.4C). FIH-1 eluates with DFO present and HPH-2 eluates regardless of DFO status had no detectable HIF-1α asparaginyl hydroxylase activity. Furthermore, hydroxylase activity of HPH-2 and FIH-1 enzymes purified in the presence of DFO were recovered when reaction buffer was supplemented with Fe(II) (data not shown). However, purification of neither HPH-2 nor FIH-1 in the presence of DFO resulted in co-purification of additional protein(s) than in its absence (Fig. 4.4D).

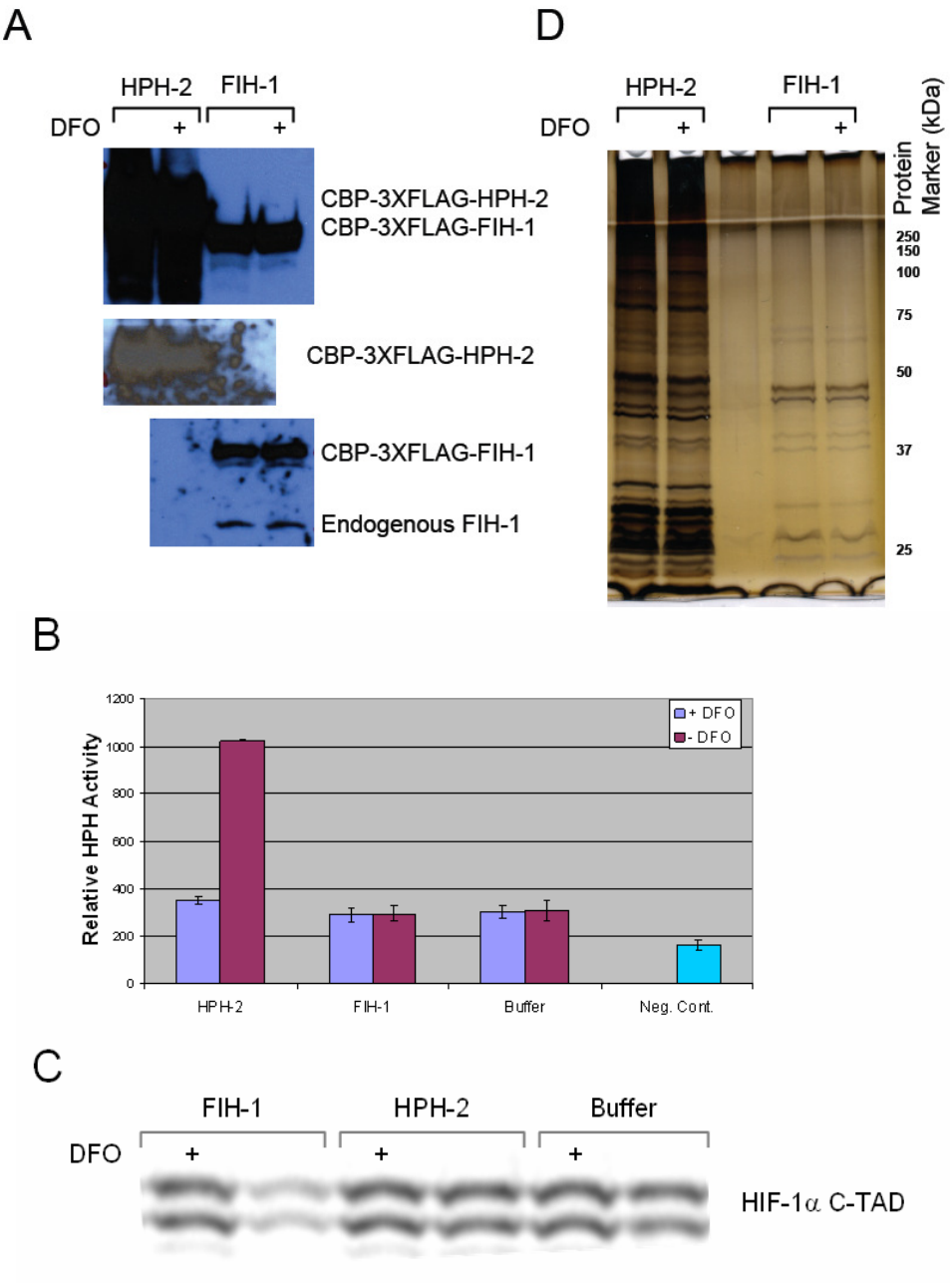


Figure 4.4 Tandem affinity purification of HPH-2 and FIH-1 in the presence of iron chelator.
Purifications NTAP-3XFLAG-HPH-2 and NTAP-3XFLAG-FIH-1 from 293 stable cell lines were done in presence or absence of 100 μM DFO.
(A) Final TAP eluates were separated by SDS PAGE and immunoblotted with α-FLAG M2 (upper panel), α-HPH2 (middle panel) and α-FIH-1 antibodies (lower panel).
(B, C) HIF prolyl and asparaginyl hydroxylase activity of each sample were assayed with HPH ELISA-based (B) and FIH-1 GST pull-down-based assays (C), respectively.
(D) Proteins in final eluates were separated by SDS PAGE and silver stained. Molecular weight of protein size markers are indicated next to the gel.

TRiC chaperone complex co-purifies with HPH-1

Masses of peptide fragments derived from 90 kDa HPH-1 co-purified protein band were consistent with that of Heat Shock Protein 90 (HSP90). However, 60 kDa and 50 kDa doublet bands matched with subunits of the TCP-1 ring complex (TRiC; also known as chaperonin containing TCP-1 (CCT)). TRiC is a chaperonin complex of eight subunits (Dunn, Melville et al. 2001), seven of which were identified by mass spectrometric analysis of the peptides derived from 60 kDa and 50 kDa proteins bands. Peptides of triple protein band around 50 kDa matched with β-tubulin, antibody heavy chain, heterogenous nuclear ribonucleoprotein protein H1 (hnRNP H1), and RuvB-like 2 proteins. As expected the 33 kDa band was from HPH-1 protein (CBP-3XFLAG-HPH-1) whereas 30, 28, and 26 kDa bands were of HPH-1 protein, perhaps a degradation product of some kind, ribosomal proteins, or antibody light chain. Take together, this data indicate that exogenous HPH-1 protein is primarily associated with the TRiC chaperonin complex. Consistent with our result, the TRiC complex was co-immunoprecipitated with HPH-1 from U20S stable cell lines and it was implicated in folding of HPH-1 protein *in vivo* (Masson, Appelhoff et al. 2004).

HPH-3 and FIH-1 form simple protein complexes

Analysis of peptides derived from HPH-3 co-purified protein bands led to assignment of HPH-3 to all of the bands submitted for protein identification from HPH-3 eluate (Table 4.4). The 50 kDa band in HPH-3 eluate is consistent with the size of CBP-3XFLAG-HPH-3 and 27, 17, 15, and 10 kDa bands with degradation products of it. These data suggest that

either HPH-3 does not stably associate with other proteins or the NTAP-3XFLAG-tag interferes with its interactions.

For FIH-1 co-purified bands, the 50 kDa band is of antibody heavy chain or serum albumin coming from antibodies used for affinity purification, or tubulin-α 6, a contaminating protein which is abundant in cells. The 45 kDa band is of FIH-1 and this fits well with the expected size of CBP-3XFLAG-FIH-1 protein. The 40 kDa band is of either endogenous FIH-1 or Methylosome Protein 50 (MEP50), the 33 kDa band is of a hypothetical protein, and the 28 kDa band is of antibody light chain (Table 4.5). Comparison of the FIH-1 co-purified proteins from HeLa (Table 4.1) and 293 cells (Table 4.5) indicates that FIH-1 forms a dimer, which does not seem to tightly associate with other proteins *in vivo*.

HPH-2 co-purifies multiple proteins

Proteins that matched peptides derived from HPH-2 co-purified protein bands are listed in Table 4.3. Low molecular weight (33, 28, and 26 kDa) protein bands were assigned mostly to ribosomal proteins, HPH-2 protein (perhaps degradation products of the CBP-3XFLAG-HPH-2 protein), and antibody light chain. Consistent with calculated molecular weight of CBP-3XFLAG-HPH-2 protein, the 55 kDa protein was assigned as HPH-2 protein. Interestingly, the other protein bands (110, 85, 65, and 50 kDa) were assigned to proteins involved in RNA splicing (i.e. nucleolin and DRBP76α – a splice variant of ILF3), transcriptional regulation (i.e. TRIM28), DNA replication (i.e. RPA1 and RPA2), and proteins with enzymatic activities (i.e. arginine methyltransferase PRMT5, DNA helicases

Ku80, DDX50, and DDX5, and peptidyl-prolyl isomerase FKBP8). Surprisingly, most of these proteins are nuclear proteins which suggest that in nucleus, HPH-2 may serve additional functions besides regulation of HIF.

Many of the aforementioned candidate HPH-2 interacting proteins were cloned into mammalian and bacterial expression vectors for further studies. Even though they were not identified as HPH-2 interacting proteins with tandem affinity purification method, we also cloned and included PRMT1 and RPA3 in our studies. PRMT1 was identified as a HPH-2 interacting protein in yeast two-hybrid screen (Chapter 1) though the interaction was not confirmed by other methods. PRMT1, like PRMT5, is a protein arginine methyltransferase; however, unlike PRMT5 (symmetric dimethylation), PRMT1 asymmetrically dimethylates arginine residues(Bedford and Richard 2005). The third subunit of Replication Protein A complex (RPA3) was included in our studies since the other two subunits (RPA1 and RPA2) were among the candidate HPH-2 interacting proteins.

Interactions between HPH-2 and the candidate interacting proteins were tested by co-immunoprecipitation of transiently expressed 3XFLAG-tagged proteins together with endogenous HPH-2 (Figs. 4.5A and 4.5B). FKBP8, PRMT1, IGF2BP1, TRIM28, RPA1, and DDX50 were precipitated with two different α-HPH-2 immune sera but less so with pre-immune serum. RPA3 and Ku80 were precipitated with α-HPH-2 #1 but not with α-HPH-2 #2 immune serum or the pre-immune serum suggesting that RPA3 and Ku80 binding block the HPH-2 epitope of the latter immune serum. Interactions with other candidates tested with the same setup were either inconclusive due to non-specific binding or non-detectable as exemplified with DDX5 and nucleolin, respectively (Fig. 4.5A).

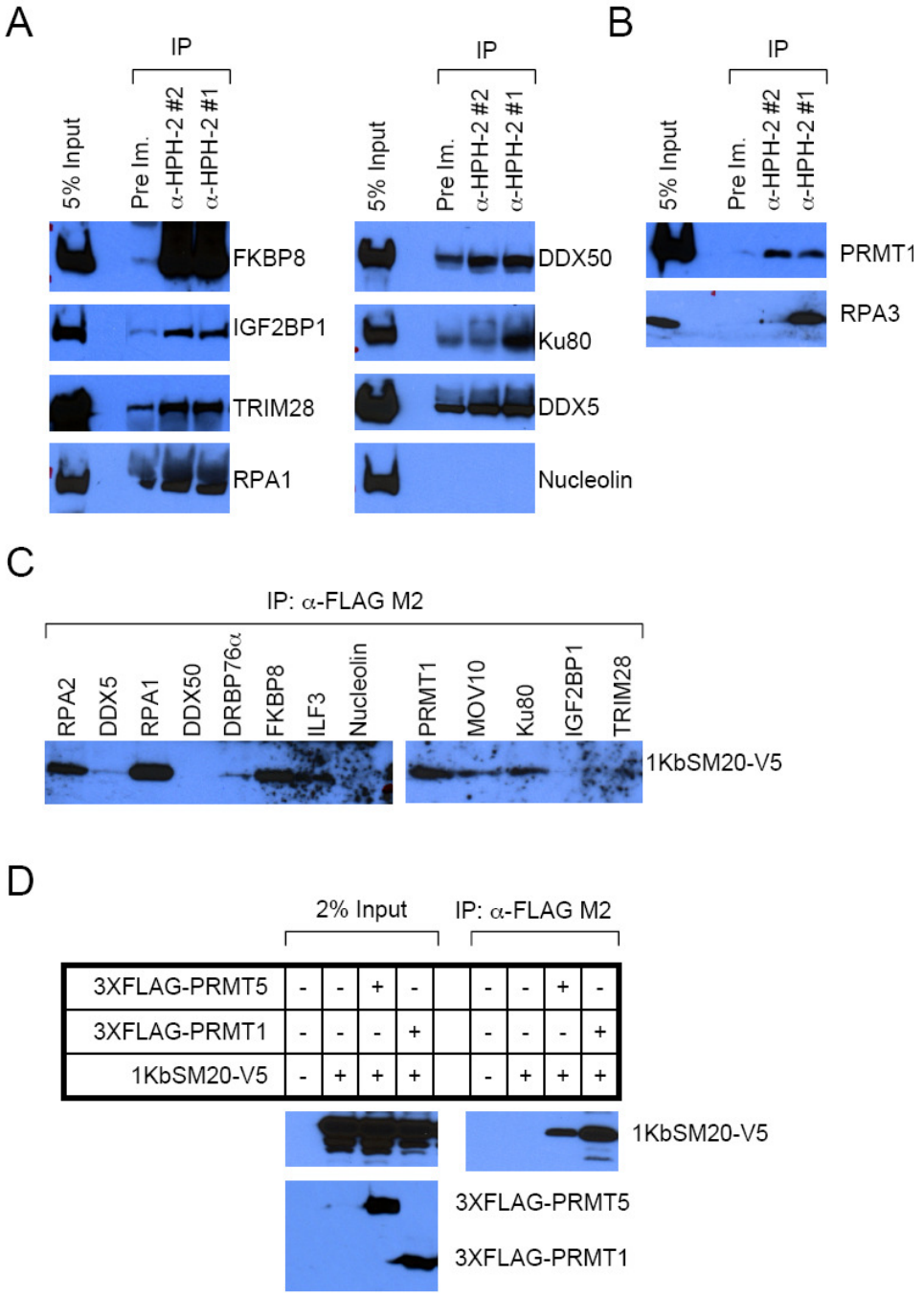


Figure 4.5 Confirmation of interaction between HPH-2 and candidate HPH-2 interacting proteins.
HPH-2 associated proteins purified with TAP method (A) or other candidates of HPH-2 interaction partners (B) were transiently expressed as N-terminal 3XFLAG-tagged proteins and immunoprecipitated with two different α-HPH-2 immune serum or a pre-immune serum. Co-precipitated proteins and 5% of the input lysates were analyzed by western blot using α-FLAG M2 antibody.
(C, D) V5-tagged 1KbSM20 protein co-expressed with the 3XFLAG-tagged proteins was immunoprecipitated with α-FLAG M2 antibody and detected with α-V5 antibody in western blot analysis.

In a reciprocal co-immunoprecipitation experiment, interaction between transiently co-expressed 3XFLAG-tagged proteins and V5-tagged 1KbSM20 were tested (Figs. 4.5C and 4.5D). Co-precipitation of 1KbSM20-V5 protein with α-FLAG M2 antibody in the presence of 3XFLAG-tagged RPA2, RPA1, FKBP8, ILF3, PRMT1, MOV10, and Ku80 (Fig. 4.5C), and PRMT1 and PRMT5 (Fig. 4.5D) indicated a positive protein-protein interaction between these proteins and HPH-2. Detection of HPH-2 interaction in one but not the other co-immunoprecipitation studies might be attributed to differences in protein surfaces used for antibody binding and protein-protein interactions.

Nucleolin is an abundant nuclear phosphoprotein implicated in almost every nuclear process such as DNA replication, chromatin remodeling, and transcriptional regulation (Tuteja and Tuteja 1998). Nucleolin has been shown to interact with many nuclear proteins including the Replication Protein A complex (Kim, Dimitrova et al. 2005) components of which are HPH-2 interacting proteins (Fig. 4.5). Therefore, it is possible that association of nucleolin with HPH-2 could have been mediated by the RPA complex in tandem affinity purification, which may explain why we could not detect an interaction between the two proteins in our co-expression/co-immunoprecipitation studies (Figs. 4.5A and 4.5C).

In summary, we have confirmed a positive interaction between HPH-2 and the following proteins by at least one independent co-immunoprecipitation experiment; MOV10, TRIM28, Ku80, DDX50, RPA1, PRMT5, IGF2BP1, FKBP8, and RPA2 –as indicated in Co-IP column of Table 4.3–, and ILF3, PRMT1, and RPA3 proteins.

Candidate HPH-2 interacting proteins do not affect HPH-2 activity

One of the potential functions of the candidate HPH-2 interacting proteins may be to serve as regulators of HPH-2 enzymatic activity. Thus, we assayed their affect on HPH-2 hydroxylase activity in an ELISA-based assay, where hydroxylation of HIF-1α peptide substrate was used to measure HIF prolyl hydroxylase (HPH) activity. Activities of recombinant HPH-2C (catalytic C-terminal domain of HPH-2, a.acids 181-426), 1KbSM20 (an HPH-2 isoform missing a.acids 76-178), and HPH-2 (full-lentgh protein a.acids 1-426) were not affected by MBP-ILF3, -DRBP76α, and -DDX50 fusion proteins (Fig. 4.6A). Likewise, activity of recombinant HPH-2 remained constant when assayed in the presence of MBP, MBP-RPA2, or MBP-RPA3; however it was slightly attenuated by MBP-FKBP8 protein (Fig. 4.6B). *In vivo* effects of the candidate HPH-2 interacting proteins were tested by transient transfections. Lysates were prepared from 293 cells transiently transfected with expression constructs of HPH-2 interacting proteins or empty vector and endogenous HIF prolyl hydroxylase activity was measured by an ELISA-based assay. With the exceptions of TRIM28 and Nucleolin, which only in hypoxic lysates slightly reduced, and FKBP8, which slightly induced HPH activity, other HPH-2 interacting proteins were found not to affect endogenous HPH activity. A 293 stable cell line where expression (by HIF-responsive Nip3 promoter) and stability (by HIF-1α ODD fusion) of a luciferase reporter is tightly controlled by HPH activity was also used to test effect of HPH-2 interacting proteins (Fig. 4.6D). Although transient expression of RPA2, DRBP76α, MOV10, and Ku80 proteins did not alter luciferase activity under normoxic or hypoxic conditions, FKBP8 induced luciferase activity under hypoxia.

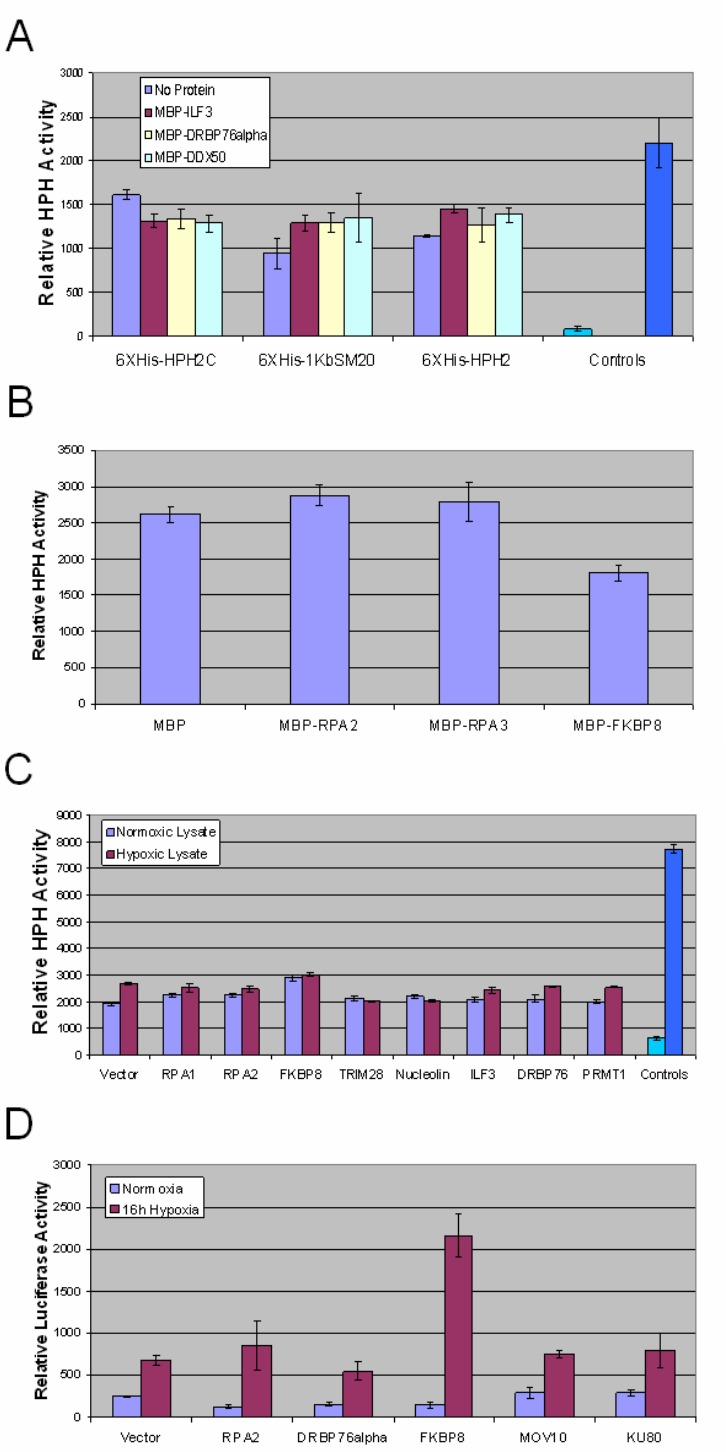


Figure 4.6 HPH-2 interacting proteins do not affect HIF prolyl hydroxylase activity.
HIF prolyl hydroxylase (HPH) activities were measured with an ELISA-based assay for recombinant 6XHis-HPH-2C, 6XHis-1KbSM20, and 6XHis-HPH-2 proteins incubated with MBP-ILF3, -DRBP76α, -DDX50

fusion proteins or without any protein (A), recombinant 6XHis-HPH-2 protein incubated with MBP-RPA2, -RPA1, or -FKBP8 fusion proteins or MBP alone (B), and for endogenous HPH proteins in lysates of 293 cells expressing HPH-2 interacting proteins (C). Negative (HIF-1α peptide substrate without enzyme) and positive (synthetic hydroxylated HIF-1α peptide) controls of HPH ELISA assay are shown with cyan and blue colored bars, respectively. (D) 293 cells stably transfected with Nip3 promoter-Luciferase-HIF-1αODD construct were transiently transfected with indicated HPH-2 interacting protein expression constructs or empty vector and luciferase activity of cells grown under normoxia and 16 h hypoxia was measured 36-40 h post-transfection.

FKBP8 is not a HPH-2 substrate

The immunosuppressant drug FK506-binding protein 8 (FKBP8 also known as FKBP38) is a peptidyl prolyl isomerase (PPIase) (Edlich, Weiwad et al. 2005), which is involved in stabilization and mitochondrial translocation of the anti-apoptotic Bcl-2 protein which in turn prevents initiation of apoptosis (Shirane and Nakayama 2003; Kang, Feng et al. 2005). Effects of FKBP8 on HPH-2 activity and hypoxic response –repression of hydroxylase activity of recombinant HPH-2 (Fig. 4.6B), induction of Nip3-Luciferase-HIF-1α ODD reporter (Fig. 4.6D), and induction of endogenous HPH activity (Fig. 4.6C)– were all consistent with FKBP8 being a HPH-2 substrate. Furthermore, the FKBP8 protein sequence has several proline containing fragments, which resemble hydroxylation motifs recognized by HPH enzymes on HIF-α subunits. To test whether HPH-2 hydroxylates FKBP8, full-length (a. acids 1-413) or C-terminal deleted (a. acids 1-210) recombinant FKBP8 proteins were incubated with 1KbSM20 enzyme, where hydroxylation was monitored by decarboxylation of the [^{14}C]-2-oxoglutarate co-substrate releasing [^{14}C]-CO_2. Compared to enzyme alone, neither MBP nor MBP-fusions of FKBP8 induced [^{14}C]-CO_2 release, while a true HPH substrate (HIF-1α peptide) led to a robust increase, (Fig. 4.7) indicating that FKBP8 is not a HPH-2 substrate.

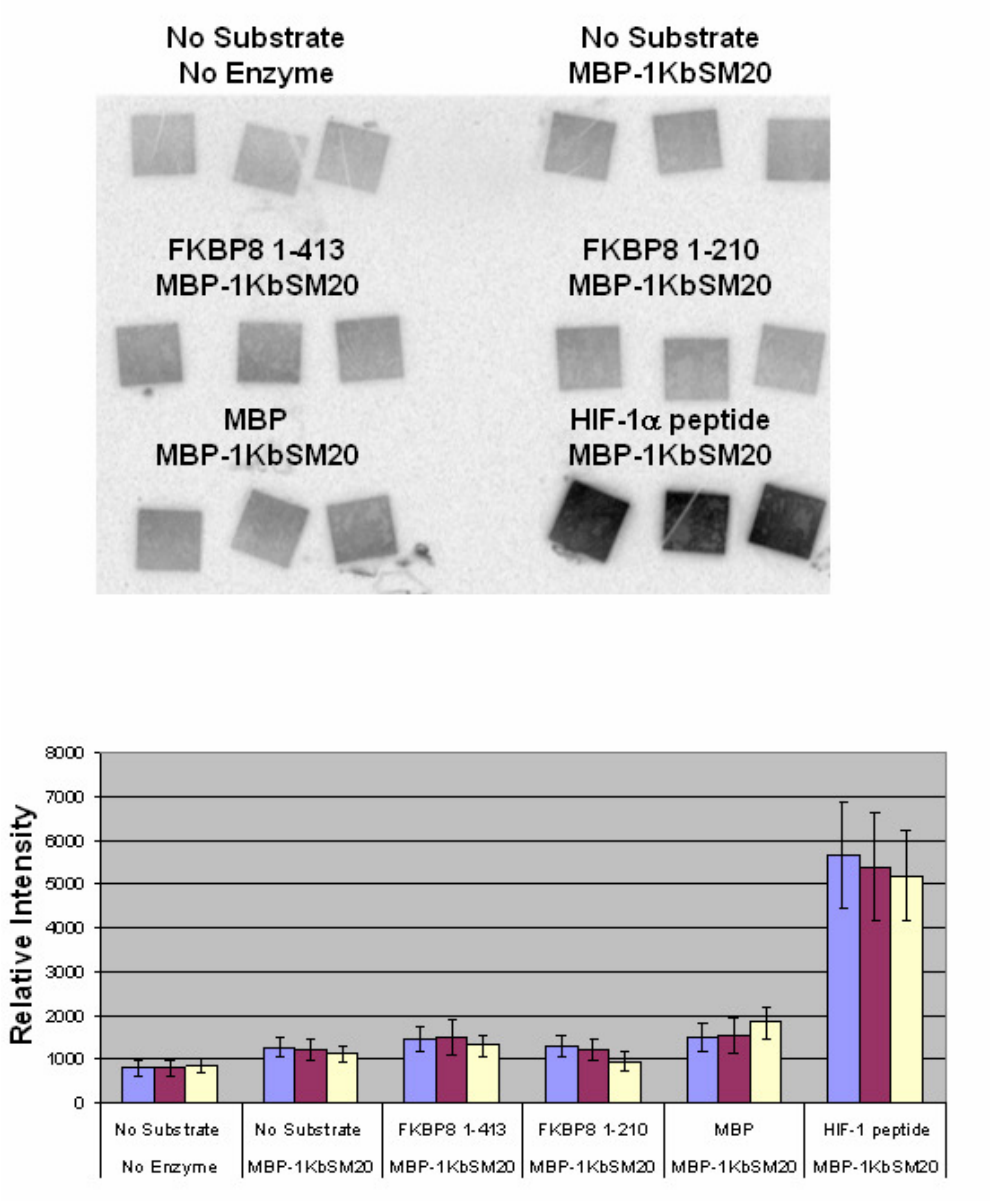


Figure 4.7 HPH-2 does not hydroxylate FKBP8.
Hydroxylation of recombinant FKBP8 protein (full length a.acids 1-413 and C-terminal deletion a.acids 1-210) by MBP-1KbSM20 fusion protein in the presence of [^{14}C]-2-oxoglutarate co-substrate was measured by generation of [^{14}C]-CO2, which was captured on Whatmann paper and exposed to PhosphorImager screen. Negative (no enzyme/no substrate, enzyme/no substrate, or enzyme/MBP protein) and positive (enzyme/HIF-1α peptide substrate) controls for 2-Oxoglutarate Decarboxylation Assay are shown. Intensity of radioactivity for each sample was quantified with ImageQuant program and shown in bar graph.

HPH-2 does not hydroxylate mass spectrometry identified interacting proteins

Analysis of other candidate HPH-2 interacting protein sequences revealed proline containing sequences that are similar to the HIF-α hydroxylation motif (LXXLAP) such as the LXXLFP sequence found in ILF3 and Ku80 proteins. Therefore, hydroxylation of all the other candidate interacting proteins by HPH-2 was tested as well. HPH-2 interacting proteins ILF3, DRBP76α, and DDX50 were recombinantly expressed and purified using a bacterial MBP-fusion protein system (Fig. 4.8A) and assayed for hydroxylation as previously described for FKBP8. As seen with FKBP8, these other HPH-2 interacting proteins did not lead to any detectable increase in [^{14}C]-CO_2 release compared to controls lacking enzyme, while HIF-1α peptide substrate used at a similar concentration was efficiently hydroxylated (Fig. 4.8B). One possible explanation for why these proteins might have not been hydroxylated *in vitro* is the absence of post-translational modifications that occur in mammalian cells but not in the recombinant bacteria system. However, the lack of an effect on luciferase reporter in mammalian cells (Fig. 4.6D), where one would expect substrates to induce reporter gene expression by overwhelming the endogenous HPH enzymes thereby causing stabilization/activation of HIF, argues against this possibility and suggests that these proteins do not serve as HPH-2 substrates either *in vitro* or *in vivo*.

HPH-2 is methylated by PRMT1

In the previously mentioned TAP purification and yeast two-hybrid screen, two of the HPH-2 interacting proteins were identified as protein arginine methyltransferases (PRMTs); PRMT1 from yeast two-hybrid screen (Chapter 1) and PRMT5 from tandem affinity

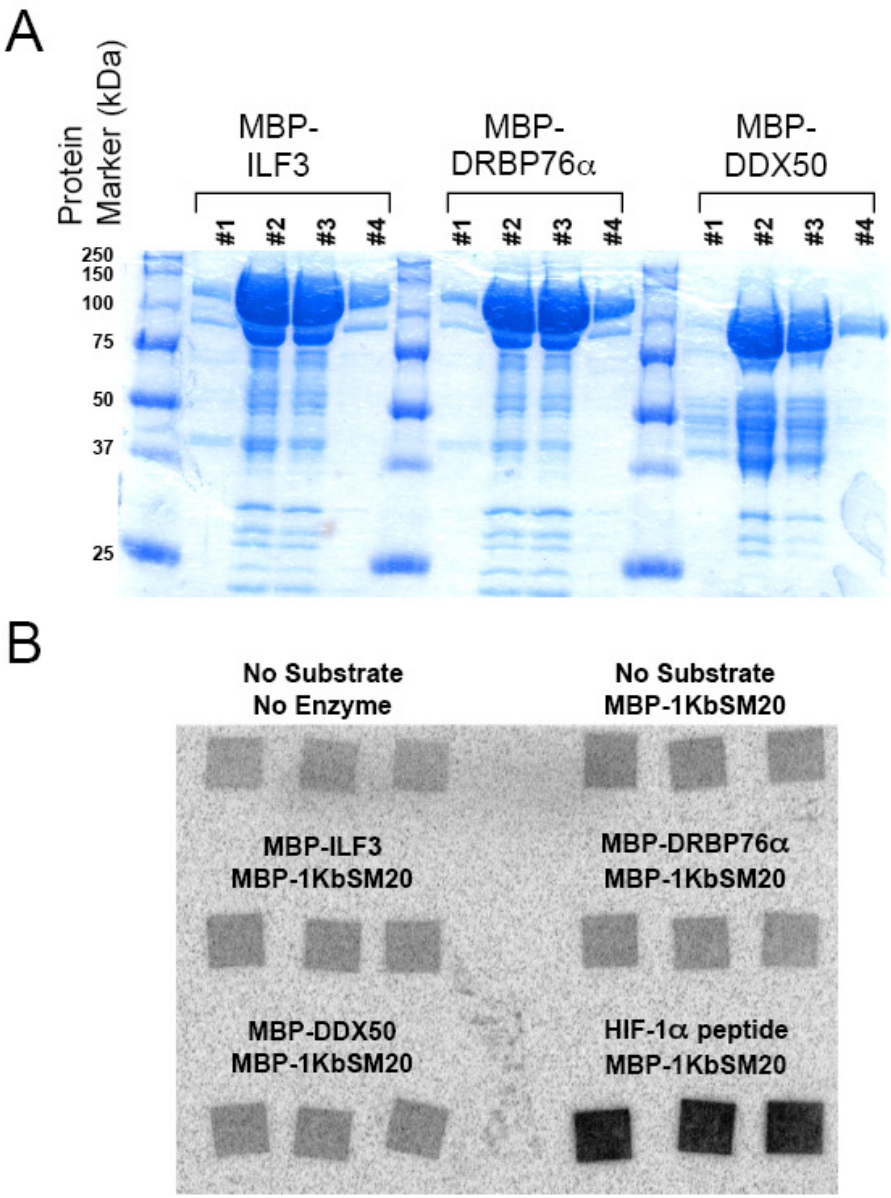


Figure 4.8 Hydroxylation of HPH-2 interacting proteins by HPH-2.
(A) Purified recombinant MBP-ILF3, -DRBP76α, and –DDX50 proteins were resolved by SDS PAGE and stained with coomassie blue.
(B) Hydroxylation of recombinant proteins (MBP-ILF3, -DRBP76α, and –DDX50) by MBP-1KbSM20 fusion protein in the presence of [^{14}C]-2-oxoglutarate co-substrate was measured by generation of [^{14}C]-CO2, which was captured on Whatmann paper and exposed to PhosphorImager screen. Negative (no enzyme/no substrate and enzyme/no substrate) and positive (enzyme/HIF-1α peptide substrate) controls for 2-Oxoglutarate Decarboxylation Assay are shown.

purification of HPH-2 (Table 4.3). Both of these PRMT enzymes dimethylate arginine side-chains in target proteins by utilizing S-adenosyl-L-methionine as the methyl group donor. However, these enzymes differ in their activity as PRMT1 generates asymmetric whereas PRMT5 generates symmetric dimethylated arginine residues. Interaction of HPH-2 with two different PRMT enzymes led us to hypothesize that HPH-2 might be a target for methylation. To test this possibility, methylation assays were carried out in the presence of S-adenosyl-L-[Methyl-^{14}C]-methionine using wild-type and catalytically inactive mutant PRMT1 and PRMT5 enzymes. Bacterially expressed wild-type PRMT1 but neither mutated PRMT1 nor wild-type PRMT5 led to [^{14}C]-methylation of 1KbSM20 *in vitro* (Fig. 4.9A). Similarly, a known substrate of PRMT enzymes, Myelin Basic Protein (MyBP), was only methylated by wild-type PRMT1 enzyme. Based on the lack of MyBP methylation with recombinant PRMT5 we concluded that recombinant PRMT5 was inactive. Thus, to obtain purified active PRMT5 capable of methylation and to test whether HPH-2 is methylated by PRMT5 or not, PRMT1 and PRMT5 were transiently expressed as 3XFLAG-tagged proteins in 293 cells and affinity purified with α-FLAG M2-agarose resin. Precipitates of empty vector-transfected cells resulted in a background methylation of MyBP; however, it was enhanced when MyBP was incubated with immunoprecipitates of wild-type PRMT1 and PRMT5 (Fig. 4.9B). Mutant PRMT1 and PRMT5 immunoprecipitates had only a basal level of methyltransferase activity. In contrast, only wild-type PRMT1 precipitate was able to methylate 1KbSM20. Methylation of HPH-2C in addition to 1KbSM20 and full-length HPH-2 proteins by PRMT1 immunoprecipitates indicated that the target arginine residue(s) reside(s) within the hydroxylase domain (Fig. 4.9C). In order to assure the specificity of HPH-2 methylation,

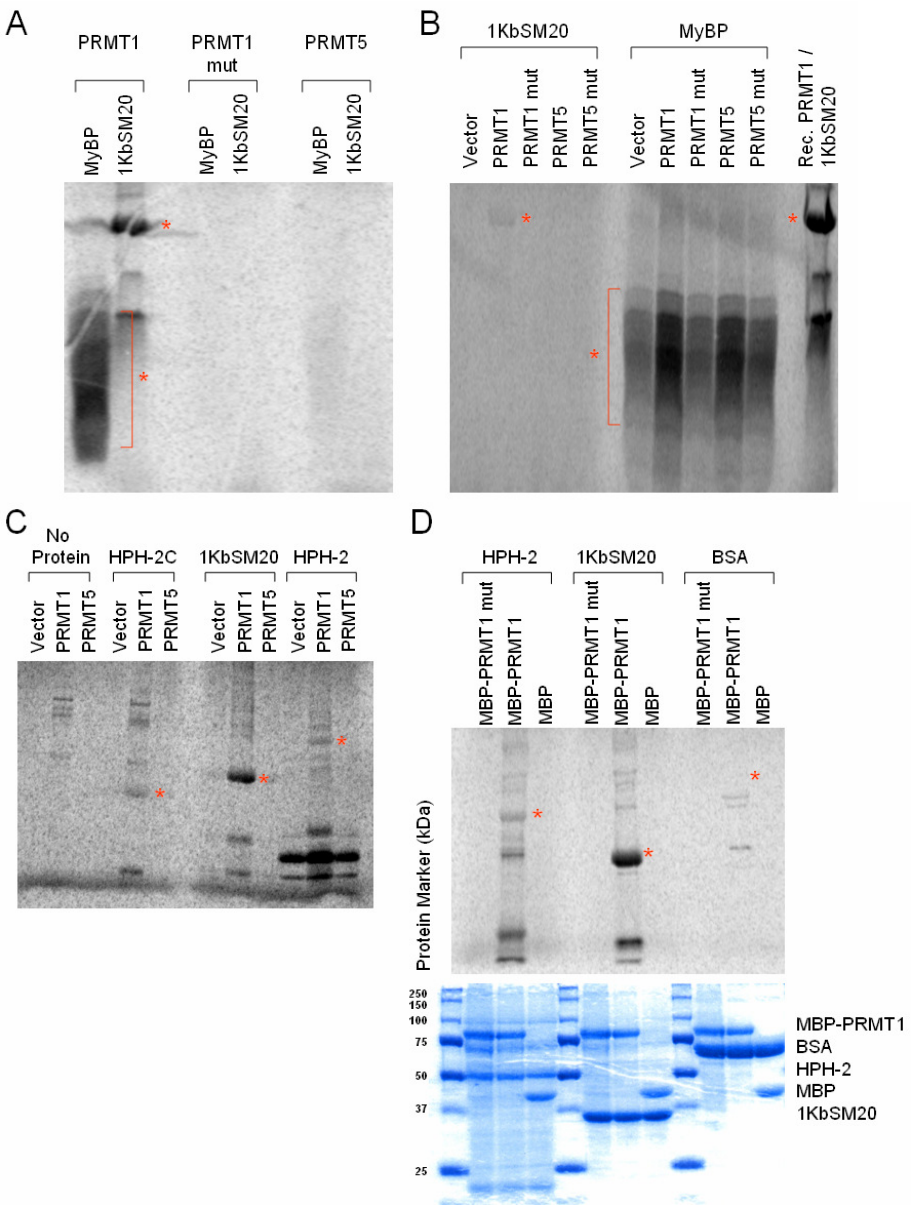


Figure 4.9 PRMT1 methylates hydroxylase domain of HPH-2 *in vitro*.
(A) Methylation of 6XHis-1KbSM20 and Myelin Basic Protein (MyBP) by MBP-PRMT1, -PRMT1 mutant, and -PRMT5 recombinant proteins in the presence of S-Adenosyl-L-[^{14}C-Methyl]-Methionine ([^{14}C]-SAM) co-substrate was detected by exposure of gel to PhosphorImager screen following SDS PAGE of each methylation reaction.
(B) Methylation of 6XHis-1KbSM20 protein and Myelin Basic Protein (MyBP) by 3XFLAG-PRMT1, -PRMT1 mutant, -PRMT5, and –PRMT5 mutant proteins purified from transiently transfected 293 cells.
(C) Methylation of 6XHis-HPH-2C, 6XHis-1KbSM20, and 6XHis-HPH-2recombinant proteins by 3XFLAG-PRMT1 and 3XFLAG-PRMT5 proteins purified from transiently transfected 293 cells.
(D) Methylation of 6XHis-1KbSM20, 6XHis-HPH-2, and Bovine Serum Albumin (BSA) proteins by recombinant MBP, MBP-PRMT1, and MBP-PRMT1 mutant proteins. Coomassie blue stained gel shown in lower panel. Location of substrate proteins on PhosphorImager screen scans are indicated by red asterisks.

Bovine Serum Albumin (BSA) was incubated with recombinant wild-type and mutant PRMT1 protein neither of which methylated BSA (Fig. 4.9D). Under the same conditions, HPH-2 and 1KbSM20 proteins were readily methylated by wild-type PRMT1.

PRMT1 methylates HPH-2 and HPH-3 but not FIH-1

It was observed that the methylation of HPH-2 mediated by PRMT1 occurred within the hydroxylase domain of HPH-2 (Fig. 4.9C), which is conserved among HPH enzymes (Fig. 4.11B). This novel finding suggested that PRMT1 might target other HPH enzymes as well and as expected, when PRMT1 recombinant enzyme was incubated with MBP-fusions of 1KbSM20, HPH-2, and HPH-3, it was found that these HPH enzymes were methylated *in vitro* (Fig. 4.10). On the contrary, neither MBP nor MBP-FIH-1 proteins were methylated *in vitro* establishing HIF prolyl hydroxylases as novel specific substrates of PRMT1. Methylation of HPH-1 could not be tested due to low levels of bacterial expression and aggregation of the purified recombinant protein in solution.

Arg295 and Lys297 of HPH-2 are critical for methylation

In order to identify the methylation target site, recombinant 6XHis-1KbSM20 protein methylated in the presence non-radiolabeled SAM by PRMT1 was submitted to the Protein Chemistry Technology Center, UT Southwestern Medical Center at Dallas. Nano-HPLC/MS/MS analysis of tryptic peptides indicated a mass difference of 28 Da, consistent with dimethylation, on two peptides derived from hydroxylase domain of HPH-2; 345-IFPEGKAQFADIEPK-359 and 291-KINGRTKAMVACYPGNGTGY-310 peptides.

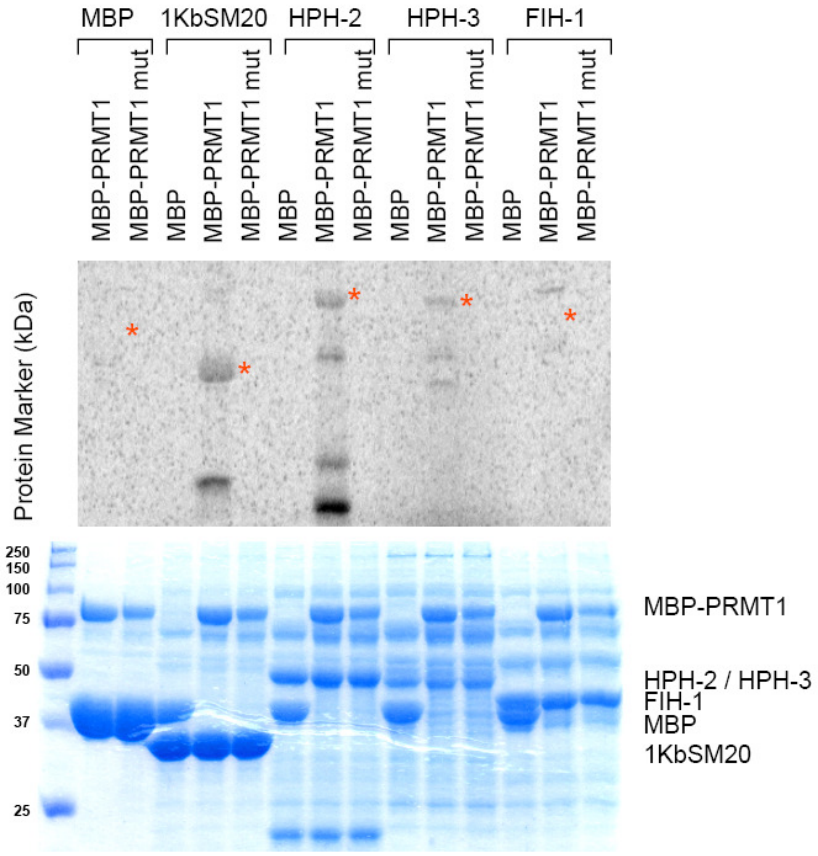
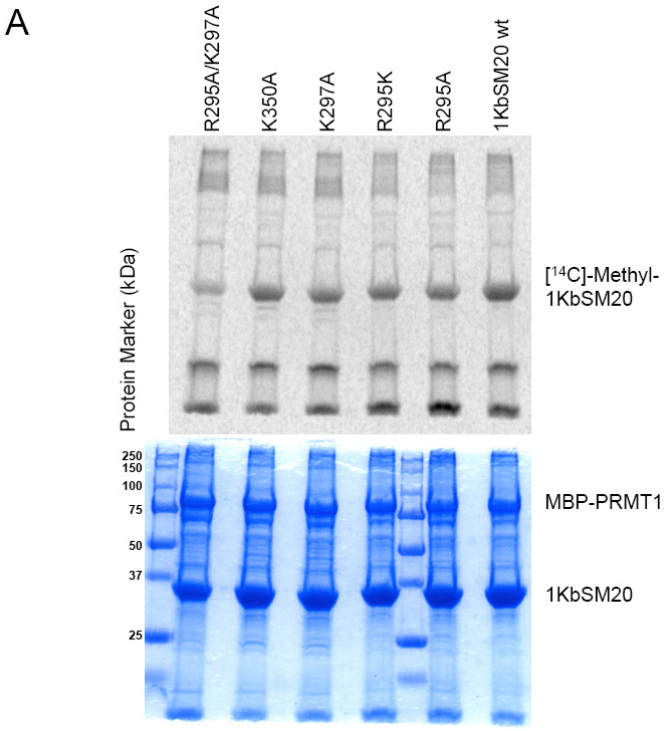


Figure 4.10 PRMT1 methylates HPH-2 and HPH-3 but not FIH-1.
Methylation of MBP (negative control), 6XHis-1KbSM20, 6XHis-HPH-2, 6XHis-HPH-3, and 6XHis-FIH-1 recombinant proteins by MBP, MBP-PRMT1, and MBP-PRMT1 mutant proteins was detected by exposure of gel to PhosphorImager screen following SDS PAGE. Coomassie blue stained gel is shown in lower panel.

Surprisingly, dimethylation of 345-IFPEGKAQFADIEPK-359 peptide was detected on Lys350 despite the known arginine specificity of PRMT1 methyltransferase (Tang, Kao et al. 2000). Fragmentation pattern of the 291-KINGRTKAMVACYPGNGTGY-310 peptide was indicative of dimethylation at either Arg295 or Lys297; however, it did not have enough resolution to determine which one of the two sites was dimethylated.

To test the site specificity of this methylation, 1KbSM20 was mutated at each site and assayed for methylation by PRMT1. Single point mutants (R295A, R295K, K297A, and K350A) were methylated as efficiently as the wild-type protein (Fig. 4.11A). On the other hand, methylation of R295A/K297A double mutant was reduced significantly suggesting that Arg295 and Lys297 are critical for methylation of HPH-2 by PRMT1 and perhaps as primary methylation site(s). HPH-3, which can be methylated by PRMT1 (Fig. 4.10), contains identical residues (Arg279 and Lys281) at the corresponding positions (Fig. 4.11B). Furthermore, HPH-1 also has Arg and Lys at those positions suggesting that HPH-1 can be methylated by PRMT1 at Arg117 or Lys119.

However, it should be noted that mass spectrometric analysis of methylated 1KbSM20 was limited by the overwhelming abundance of the unmethylated protein; the best estimates for the methylation efficiency was around 3%. In addition, methylation of 1KbSM20 R295A/K297A double mutant by PRMT1 at a detectable level indicated that there are additional methylation sites, one of which might be Lys350. In order to identify these sites and at the same time overcome limitations in methylation site identification due to inefficiency of *in vitro* methylation, a future experiment where analysis of 1KbSM20 protein methylated in the presence of S-adenosyl-L-[Methyl-^{14}C]-methionine needs to be performed.



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HPH-1      1 MPLGHIMRLDLEKIALEYIVPCLHEVGFCYLDNFLGEVVGDCVLERVKQLHCTGALRDGQ
HPH-2    180 RPNGQTKPLPALKLALEYIVPCMNKHGICVVDDFLGKETGQQIGDEVRALHDTGKFTDGQ
HPH-3    165 -LMEEALPSAPERLALDYIVPCMRYYGICVKDSFLGAALGGRVLAEVEALKRGGRLRDGQ

HPH-1     61 LAGPRAGVSKRHLRGDQITWIGGNEEGCEAISFLLSLIDRLVLYCGSRLGKYYVKERSKA
HPH-2    240 LVSQKSDSSK-DIRGDKITWIEGKEPGCEIIGLLMSSMDDLIRHCNGKLGSYKINGRTKA
HPH-3    224 LVSQRAIPPR-SIRGDQIAWVEGHEPGCRSIGALMAHVDAVIRHCAGRLGSYVINGRTKA

HPH-1    121 MVACYPGNGTGYVRHVDNPNGDGRCITCIYYLNKNWDAKLHGGILRIFPEGKSFIADVEP
HPH-2    299 MVACYPGNGTGYVRHVDNPNGDGRCVTCIYYLNKDWDAKVSGGILRIFPEGKAQFADIEP
HPH-3    283 MVACYPGNGLGYVRHVDNPHGDGRCITCIYYLNQNWDVKVHGGLLQIFPEGRPVVANIEP

HPH-1    181 IFDRLLFFWSDRRNPHEVQPSYATRYAMTVWYFDAEERAEAKKKFRN--------LTRKT
HPH-2    359 KFDRLLFFWSDRRNPHEVQPAYATRYAITVWYFDADERARAKVKYLTGEKGVRVELNKPS
HPH-3    343 LFDRLLIFWSDRRNPHEVKPAYATRYAITVWYFDAKERAAAKDKYQLASG--QKGVQVPV

HPH-1    233 ESALTED-
HPH-2    419 DSVGKDVF
HPH-3    401 SQPPTPT-
```

Figure 4.11 Identification PRMT1 methylated residue of HPH-2.
(A) Methylation of wild-type and R295A, R295K, K297A, K350A, and R295A/K297A mutants of recombinant 1KbSM20 protein by MBP-PRMT1 in the presence of S-adenosyl-L-[14C-Methyl]-methionine (upper panel). Coomassie blue stained gel shown in lower panel.

(B) Sequence alignment of HPH-1, HPH-2, and HPH-3 hydroxylase domains. Dimethylation sites of HPH-2 (R295 or K297) are indicated by purple color. Catalytic Fe(II)-binding residues (HXD…H motif) and 2-oxoglutarate co-substrate coordinating arginine residue (R383) are colored with blue and green, respectively. Identical residues between all three HPH proteins are shaded with black and similar residues with gray.

Arg295 is not critical for hydroxylase activity of HPH-2

Based on the arginine specificity of PRMT methyltransferase enzymes we reasoned that Arg295 is the actual methylation target site. Therefore, HPH activity of the R295A mutant protein was compared to that of wild-type 1KbSM20 protein in ELISA-based assay where the HIF-1α ODD domain was used as substrate instead of HIF-1α peptide. The R295A mutant was neither more nor less active than the wild-type protein suggesting that Arg295 is not critical for substrate binding and hydroxylase activity (Fig. 4.12). HPH activity of the other 1KbSM20 mutants (K297A, K350A, and R295A/K297A) were not any different than the wild-type protein when corrected for the protein amount (data not shown); however, solubility of the mutant proteins seemed to be lower than the wild-type, R295A/K297A mutant protein being the least soluble one.

Methyltransferase inhibition leads to further accumulation of HIF-1α under hypoxia

To study the functional role of methylation in the hypoxia response pathway *in vivo*, a general methyltransferase inhibitor adenosine-2',3'-dialdehyde (AdOx) was employed in cell culture experiments utilizing HeLa cells. The cells were treated with increasing concentration of AdOx for 28 hours and kept either under normoxic condition or exposed to hypoxic condition for the last 12 hours. Western blot analysis of HIF-1α and HPH-2 protein levels

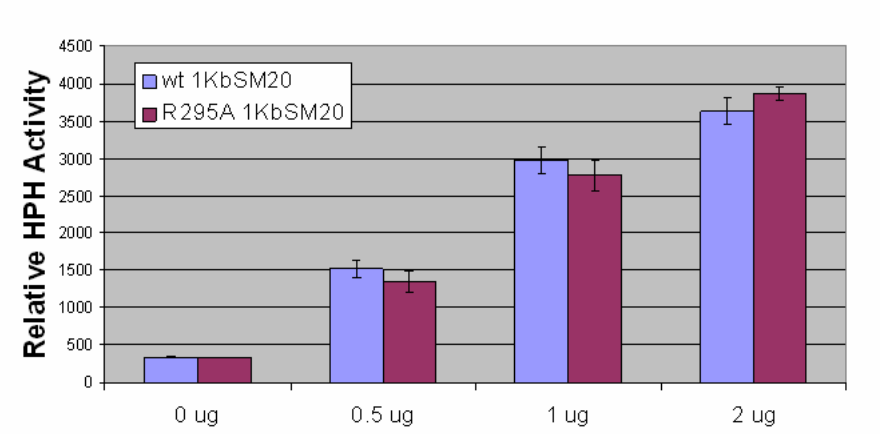

Figure 4.12 Arg295 is not critical for HPH-2 hydroxylase activity.
HIF prolyl hydroxylase activity of wild-type and R295A mutant 6XHis-1KbSM20 recombinant proteins were assayed at different amounts of enzyme with ELISA-based assay using 6XHis-HIF-1α ODD as substrate.

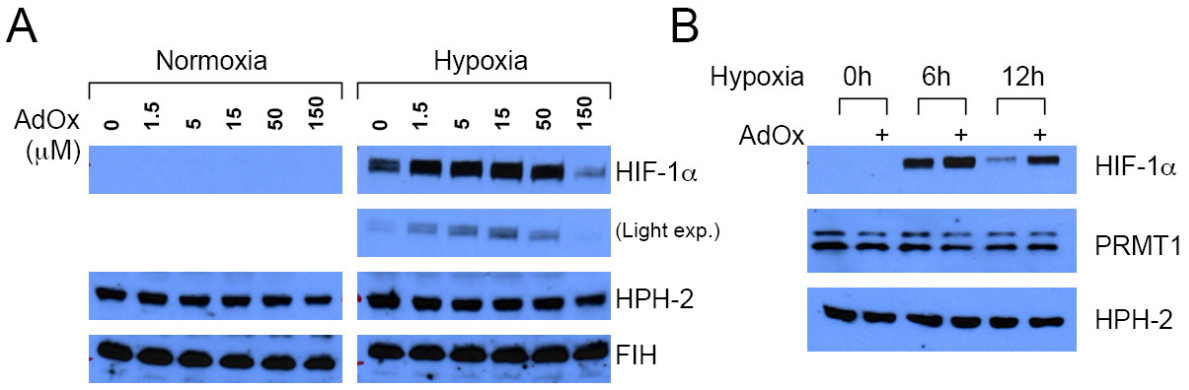

Figure 4.13 Methyltransferase inhibition further stabilizes HIF-1α under hypoxia.
(A) Lysates of HeLa cells treated with increasing concentrations of methyltransferase inhibitor AdOx were immunoblotted with α-HIF-1α, α-PRMT1, and α-HPH-2. Cells treated with AdOx for 28 hours and grown under normoxic conditions or exposed to hypoxic condition for the last 12 hours. A lighter exposure of HIF-1α blot for hypoxic lysates is also included.
(B) Lysates from 293 Luciferase-HIF-2α ODD reporter cell line treatments were immunoblotted with α-HIF-1α, α-PRMT1, and α-HPH-2 antibodies (See Fig. 4.14C for luciferase activity).

indicated that AdOx led to further accumulation of HIF-1α in a dose-dependent manner up to 50 μM while a three-fold higher concentration of AdOx destabilized HIF-1α (Fig. 4.13A). AdOx treatment did not induce HIF-1α stabilization under normoxic conditions even at 50 μM concentration. Further findings demonstrated that the levels of HIF hydroxylases, HPH-2 and FIH-1, remained constant under these conditions as determined by western blot analysis. Effect of AdOx was more pronounced with longer hypoxic exposures (Fig. 4.13B). HIF-1α was accumulated to a similar level in cells untreated or treated with 15 μM AdOx and exposed to hypoxic conditions for only 6 hours. Although HIF-1α protein level was reduced in untreated cells after 12 hours of hypoxia, AdOx treated cells retained most of HIF-1α protein accumulated at 6 hours. Likewise, luciferase activity associated with the stably transfected 3XHRE-tk-luciferase (Fig. 4.14A) and CMV-luciferase-HIF-2α ODD (Fig. 4.14C) reporter constructs were induced by AdOx treatments similar to the endogenous HIF-1α protein. In these cells, neither PRMT1 nor HPH-2 protein levels were affected by the AdOx treatments (Fig. 4.13B).

AdOx induces HIF activity in a time- and dose-dependent manner

To further characterize the effects of AdOx treatment, HIF activity was monitored by luciferase reporter in HeLa 3XHRE-tk-luciferase stable cells. Cells were treated with increasing concentrations of AdOx for 12, 22, or 30 hours and exposed to hypoxic conditions for 12 hour prior to the luciferase assay. Compared to untreated cells, cells treated with AdOx for 12 hours expressed luciferase reporter at a lower level while 22 hour AdOx treated cells expressed at much higher levels (Fig. 4.14A). However, in both cases concentration of

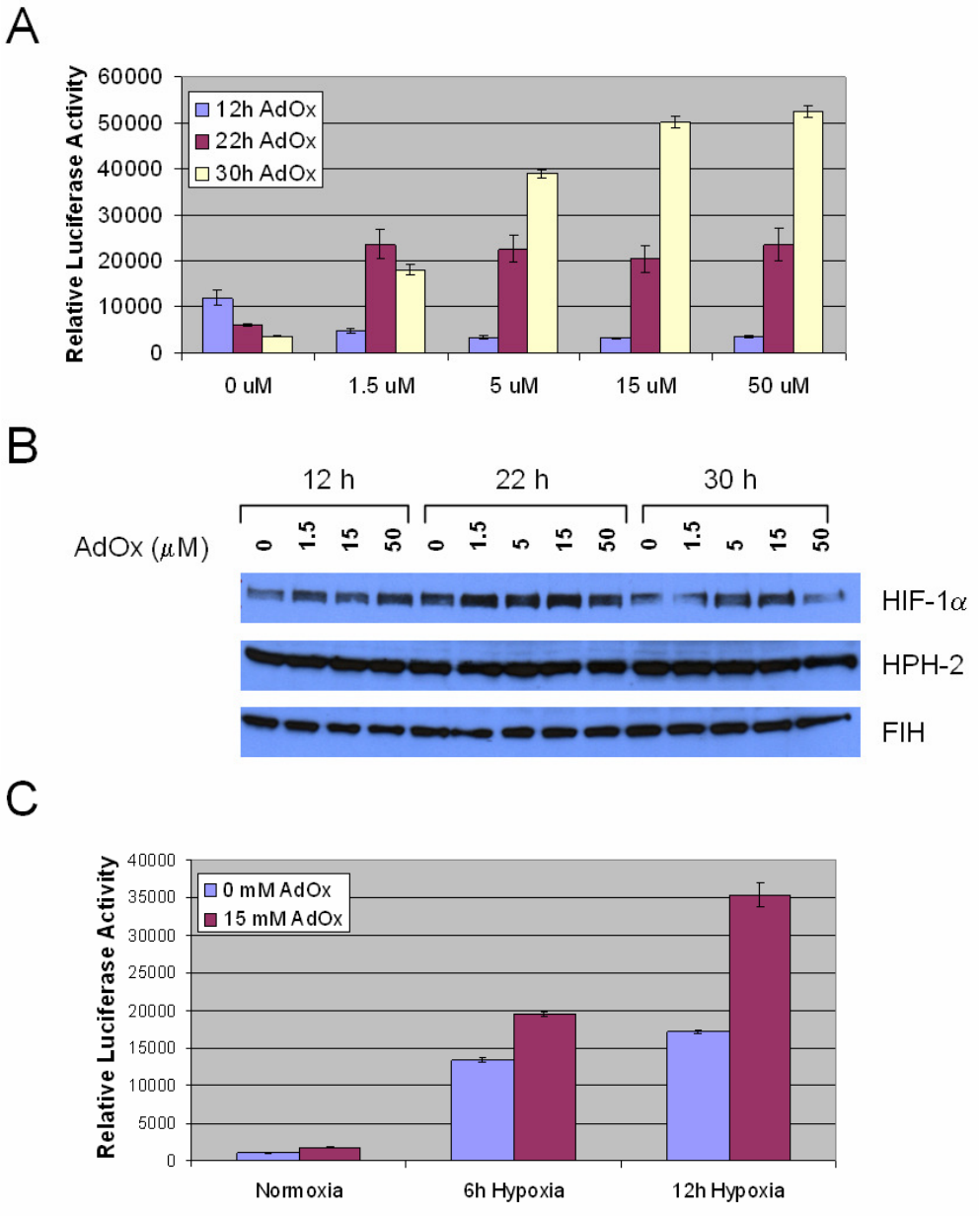

Figure 4.14 Methyltransferase inhibition induces HIF activity.
(A) Stably transfected HeLa 3XHRE-tk-Luciferase reporter cell line was treated with increasing concentrations of AdOx for 12, 22, or 30 hours and luciferase activity was measured following exposure of cells to hypoxic conditions for the last 12 hours.
(B) Lysates of AdOx-treated cells (A) were immunoblotted with α-HIF-1α, α-HPH-2, and α-FIH antibodies.
(C) Stably transfected 293 CMV-Luciferase-HIF-2α ODD reporter cell line was treated with 15 μM AdOx or left untreated (0 μM) for 28 hours and luciferase activity was measured following exposure of cells to hypoxic conditions for 0 (normoxia), 6 or 12 hours.

AdOx, at least within the range tested (1.5-50 μM), did not have an effect on luciferase reporter activity. On the contrary, AdOx induced luciferase expression in a dose-dependent manner when cells were treated with it for 30 hours. Western blot analysis of HIF-1α protein revealed that 12 and 22 hours of AdOx treatment resulted in a dose-dependent accumulation of HIF-1α whereas 30 hour treatments resulted in a dose-dependent accumulation up to 15 μM though at a lower level compared to 12 and 22 hour treatments (Fig. 4.14B). In general, 22 hour AdOx treatment resulted in accumulation of higher levels of HIF-1α than 30 hour treatment yet the luciferase expression was much higher with 30 hour treatment. All together, our data suggest a potential role for methylation in regulation of HIF transcriptional activity (Fig. 4.14) as well as HIF α-subunit stability under hypoxic conditions (Fig. 4.13). However, we are also aware of the fact that AdOx treatments might non-specifically alter HIF regulation by stressing cells, which needs to be addressed in future studies.

Discussion

The role of HIF prolyl and asparaginyl hydroxylases in regulation of HIF was discovered in 2001. Since then, these enzymes have been the focus of many studies. Mechanistic questions such as how and at what concentration of oxygen affect the function of these hydroxylases, and the regulatory effects of cellular metabolites and reactive oxygen species have been studied (Dann and Bruick 2005; Ozer and Bruick 2007). However, alternative possible modes of regulation such as interacting regulatory proteins, signaling pathways, and post-translational modifications have remained largely uninvestigated. In

order to shed some light on these potential regulatory mechanisms, we affinity purified HIF hydroxylases together with interacting proteins under near-physiological conditions.

HIF asparaginyl hydroxylase FIH-1 forms a homodimeric complex (Dann, Bruick et al. 2002; Elkins, Hewitson et al. 2003; Lee, Kim et al. 2003), and inactivated when dimerization is abolished(Lancaster, McNeill et al. 2004). Consistent with its dimeric structure, affinity purification of FIH-1 from both HeLa (Fig. 4.2) and 293 cells (Fig. 4.3) resulted in co-purification of endogenous FIH-1. This co-purification confirmed that our tandem affinity purification method was suitable for identification of functionally significant protein-protein interactions. The other common FIH-1 interacting protein identified in both affinity purifications was Methylosome Protein 50 (MEP50) (Tables 4.1 and 4.5). MEP50, a WD repeat protein, functions as the substrate specificity factor of methylosome complex for recognition of Sm proteins and it is required for efficient methylation of Sm proteins (Friesen, Wyce et al. 2002). PRMT5, the methyltransferase subunit of methylosome complex (also known as IBP72, JBP1, and SKB1) methylates Sm proteins and mediates their assembly into the spliceosome complex (Friesen, Paushkin et al. 2001). Based on these, one could imagine that activities of FIH-1 and the methylosome complex might be regulated by direct methylation and hydroxylation of the enzymes, respectively, or substrates of each enzyme might be subjected to both modifications simultaneously to give a tighter control. Therefore, we believe that the MEP50-FIH-1 interaction, which we did not have a chance to follow up on, merits further investigation.

Previously identified FIH-1 interacting proteins are: substrates- HIF-1α (Mahon, Hirota et al. 2001), p105 (NFKB1), and IκBα (Cockman, Lancaster et al. 2006) and

regulatory proteins- Siah-1 (Fukuba, Yamashita et al. 2007), pVHL, HDAC1, HDAC2, and HDAC3 (Mahon, Hirota et al. 2001). We did not detect any of these interacting proteins in tandem affinity purification of FIH-1 (Figs. 4.2 and 4.3, and Tables 4.1 and 4.5) either because these interactions are not tight enough to withstand two steps of affinity purification, or they are low abundance proteins that could have co-purified with HPH-2 but were undetectable by silver stain, or they are not real interactions. Furthermore, known substrates of FIH-1 were not among the FIH-1 co-purified proteins either. Speculating that FIH-1-substrate interactions could be enhanced with inhibition of hydroxylase activity, we affinity purified FIH-1 in the presence of DFO. Although iron chelation inactivated FIH-1 (Fig. 4.4C), it did not lead to the co-purification of additional proteins (Fig. 4.4D) suggesting that substrates loosely associate with FIH-1 or that they are present at levels beneath the detection limit of silver stain.

HIF Prolyl Hydroxylase 1 (HPH-1) was purified together with Heat Shock Protein 90 (HSP90) and subunits of TRiC chaperonin complex (Fig. 4 and Table II). HSP90 chaperone in addition to many other transcription factors also regulates HIF-1α(Minet, Mottet et al. 1999). HSP90 is required for hypoxic stabilization of HIF-1α (Minet, Mottet et al. 1999) and inhibition of HSP90 chaperone activity leads to degradation of HIF α-subunit (Mabjeesh, Post et al. 2002; Yang, Zhang et al. 2005). The TCP-1 Ring Complex (TRiC, also known as Chaperonin Containing TCP-1 (CCT)) is a cytoplasmic chaperonin complex involved in mediating protein folding. Known substrates of this complex include cytoskelatal proteins actin and tubulin, cell cycle protein cyclin E, signaling protein G α-transducin, and pVHL (Dunn, Melville et al. 2001). Assembly of pVHL into an active E3 ubiquitin ligase complex

(pVHL/Elongin B/Elongin C) is mediated by TRiC(Feldman, Thulasiraman et al. 1999). Interestingly, some of the tumor associated mutations of VHL gene abolish pVHL-TRiC interaction, emphasizing the importance of TRiC complex. Recently, HPH-1 was co-purified with TRiC chaperonin complex by others as well (Masson, Appelhoff et al. 2004). HPH-1 is likely to be another substrate of TRiC chaperonin as suggested by (Masson, Appelhoff et al. 2004). However, it is also possible that HPH-1 might be a subunit of the complex where it hydroxylates proteins to aid their folding or targets them to proteosomal degradation when they can not be folded properly. Such a function of HPH-1 needs to be tested experimentally.

In our tandem affinity purification/mass spectrometry analysis we did not detect the previously identified HPH-1 interacting proteins; MORG1, which acts as a molecular scaffold for HPH-1 (Hopfer, Hopfer et al. 2006), or SIAH1/2 E3 ubiquitin ligases, which restricts HPH-1 protein levels under hypoxia by marking HPH-1 for proteosomal degradation (Nakayama, Frew et al. 2004). This lack of co-purification can be attributed to several reasons including weak/transient interaction, blockage of interaction by NTAP-3XFLAG-tag, and low protein abundance, or these interactions of HPH-1 may be artifactual.

Analysis of the HPH-2 co-purified protein bands revealed several interesting proteins many of which are nuclear proteins involved in transcriptional regulation, DNA replication/repair, and post-translational modification of proteins (Table 4.3). TRIM28 (also known as KAP-1 and TIF1 β) is a RING and PHD finger containing nuclear co-repressor protein (Friedman, Fredericks et al. 1996), which inhibits p53 (Wang, Ivanov et al. 2005) and c-myc (Satou, Taira et al. 2001) transcription factors. Interestingly, TRIM28 was found to interact with pVHL in a yeast two-hybrid screen (Li, Wang et al. 2003). When overexpressed

alone, TRIM28 affected neither stability nor transcriptional activity of HIF; however, when co-expressed with pVHL, it repressed HIF activity without altering the stability of α-subunit. DRBP76α is a splice variant of ILF3, which is an RNA binding protein that affects transcription, mRNA processing and translation (Reichman, Muniz et al. 2002). ILF3 has also been related to regulation of methyltransferase activity of PRMT1 (Tang, Kao et al. 2000). Ku80 is an ATP-dependent DNA helicase involved in double-strand break repair (Tuteja and Tuteja 2000). DDX50 is a DEAD-box RNA helicase which enhances c-Jun target gene expression (Westermarck, Weiss et al. 2002) and has been implicated in ribosomal RNA processing and ribosome assembly (Valdez, Perlaky et al. 2002). RPA1 and RPA2, both of which were identified as HPH-2 interacting proteins, are subunits of a trimeric protein complex called Replication Protein A (Fanning, Klimovich et al. 2006). RPA is the major single-stranded DNA (ssDNA)-binding protein involved in DNA replication, repair, and recombination, where it prevents nucleolytic cleavage and aberrant folding of ssDNA. Nucleolin, which perhaps associates with HPH-2 through RPA complex, is an abundant nuclear phosphoprotein implicated in almost every nuclear process including DNA replication, chromatin remodeling, and transcriptional regulation (Tuteja and Tuteja 1998). PRMT5 (also known as JBP1 or SKB1) is a type II protein arginine methyltransferase, which symmetrically dimethylates arginine residues (Branscombe, Frankel et al. 2001). PRMT5 associates with SWI/SNF chromatin remodeling complex and involved in transcriptional repression of Myc target gene Cad (Pal, Yun et al. 2003) and tumor suppressor genes ST7 and NM23 (Pal, Vishwanath et al. 2004). FKBP8, an immunophilin family protein which binds FK-506 immunosuppressant drug, binds and anchors anti-apoptotic Bcl-2 family

proteins to mitochondria (Shirane and Nakayama 2003). Inhibition or suppression of FKBP8 induces apoptosis. Although its activation remains controversial, FKBP8 contains peptidyl prolyl cis/trans isomerase (PPIase) domain which can be activated by Ca^{+2}-Calmodulin binding(Edlich, Weiwad et al. 2005). Interestingly, neuroprotective effects of an FKBP8-specific inhibitor (N-(N',N'-dimethylcarboxamidomethyl)cycloheximide (DM-CHX)) following transient focal cerebral ischemia has been reported (Edlich, Weiwad et al. 2006). All these protein-protein interactions of HPH-2 indicate that in addition to regulation of HIF-α subunit stability (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001; Ivan, Haberberger et al. 2002) and HIF transcriptional activity (Ozer, Wu et al. 2005; To and Huang 2005), HPH-2 might be involved in many different processes some of which would require nuclear localization. We did not detect any of the previously identified HPH-2 associated proteins; HIF-α subunits (HPH substrates) (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001; Ivan, Haberberger et al. 2002), Siah1a/2 (E3 ubiquitin ligases which target HPH-1 and HPH-3 to proteosomal degradation) (Nakayama, Frew et al. 2004), OS-9 (enhancer of HPH-2 hydroxylase activity which binds both HIF-1α and HPH-2) (Baek, Mahon et al. 2005), and ING4 (Ozer, Wu et al. 2005).

None of the newly identified HPH-2 interacting proteins, except for FKBP8, had an effect on hydroxylase activity of HPH-2 either *in vitro* (Figs. 4.6A and 4.6B) or *in vivo* (Figs. 4.6C and 4.6D). Nevertheless, a caveat of these experiments should be noted, that is, the regulatory effects of these proteins may not be manifested unless these proteins reside within their endogenous protein complexes. Although this issue was addressed at least for the core RPA complex –neither co-expression nor co-incubation of RPA1, RPA2, and RPA3 proteins

affected HPH activity (data not shown)–, the possibility that HPH-2 is regulated by the nucleolin-RPA complex still remains.

One possible explanation for the repressive effects of FKBP8 was that FKBP8 could be a novel substrate of HPH-2, and thus functions by competing with substrate. However, when tested in the *in vitro* [^{14}C]-2-oxoglutarate decarboxylation assay, FKBP8 did not induce [^{14}C]-CO_2 release (Fig. 4.7). This data suggest that rather than being a substrate, FKBP8 acts as a repressor of HPH-2, which can inhibit HPH-2 activity *in vitro* (Fig. 4.6B) and activates HIF *in vivo* (Fig. 4.6D) leading to further induction of HPH-1 and HPH-2 expression (Cioffi, Liu et al. 2003; D'Angelo, Duplan et al. 2003; Marxsen, Stengel et al. 2004). The end result being a slight increase of endogenous HPH activity in FKBP8 transfected cell lysates (Fig. 4.6C). The repressive effect of FKBP8 could be mediated by stabilization of interaction between N-terminal MYND-type zinc finger and hydroxylase domain of HPH-2, which had been implicated as an autoregulatory mechanism (Choi, Lee et al. 2005). Additionally, requirement of FKBP8's peptidyl-prolyl isomerase (PPIase) activity for repression of HPH-2 activity is unknown.

Since we were unable to detect any regulatory impact of HPH-2 interacting proteins *in vivo*, we hypothesized that these proteins could function as substrates of HPH-2. Unfortunately, none of the proteins were found to be hydroxylated by HPH-2 *in vitro* (Fig. 4.8 and data not shown), even though some of the proteins contained sequences similar to LXXLAP hydroxylation motif (Masson, Willam et al. 2001; Huang, Zhao et al. 2002) (i.e. LXXLFP motif in ILF3, DRBP76α, and Ku80). This result is consistent with the lack of an effect in transient transfection experiments, where HPH substrate proteins would be expected

to induce HPH activity (Fig. 4.9C) and Nip3-Luciferase-HIF-1α ODD reporter expression (Fig. 4.9D) by overwhelming hydroxylase activity of HPH enzymes thereby leading to HIF-α stabilization. Furthermore, tandem affinity purification done in the presence of the iron chelator DFO did not result in identification of a novel HPH-2 substrate either (Fig. 4.4D), though the purified HPH-2 was inactive (Fig. 4.4B).

Since we observed an interaction between HPH-2 and two different protein arginine methyltransferases (PRMT1 and PRMT5), we suspected that instead of a direct protein-protein interaction, HPH-2 could be regulated by methylation. By using radiolabeled co-substrate (S-adenosyl-L-[^{14}C-Methyl]-methionine) we showed that PRMT1 methylates HPH-2 (Fig. 4.9A). Methylation of HPH-2C by PRMT1 (Fig. 4.9C) indicated targeting of the highly conserved hydroxylase domain (Fig. 4.11B) allowing PRMT1 to additionally methylate HPH-3 but not FIH-1, MBP, or BSA (Figs. 4.10 and 4.9D). Consistent with the prior literature, recombinant PRMT5 was inactive (Pollack, Kotenko et al. 1999) (Fig. 4.9A) and PRMT5, affinity purified from mammalian cells, had modest activity against MyBP (Pollack, Kotenko et al. 1999) but did not methylate HPH-2 (Fig. 4.9B and 4.9C). Mass spectrometry and mutational analysis indicated that HPH-2 Arg295 and Lys297 residues were significant for methylation by PRMT1. We believe that Arg295 is the primary site of methylation; however, in its absence alternative sites could be methylated as observed for R295A and R295A/K297A mutants (Fig. 4.11A). Conservation of identical residues at similar positions on HPH-3, which can be methylated by PRMT1 (Fig. 4.10), and HPH-1 suggest that all three HPH enzymes might be methylated at similar positions. Taken together, all of these data establish HPH enzymes as novel substrates of PRMT1 enzymes and suggests

a distinctive role for PRMT1 in regulation of HPH enzymes. A similar modification of FIH-1 can not be ruled out, since FIH-1 potentially interacts with a component of methylosome complex (MEP50, Tables 4.1 and 4.5), which contains PRMT5.

Efficiency of HPH-2 methylation by PRMT1 was very low; ~3% as judged by the ratio of methylated to unmethylated peptides in mass spectrometry. This might explain why we did not observe any difference between HIF prolyl hydroxylase activity of methylated and non-methylated HPH-2 recombinant proteins (data not shown). Likewise, transient overexpression of PRMT1 and PRMT5 did not affect HIF response (data not shown) which may simply be due to inadequate levels of endogenous SAM to support methyltransferase activity of overexpressed enzymes.

Moreover, *in vivo* methylation of HPH-2 was tested by immunoprecipitation of HPH-2 from cells incubated with S-adenosyl-L-[^{14}C-Methyl]-methionine. Radiolabeling of precipitated HPH-2 was not detectable (data not shown) suggesting one of two things; either methylation of HPH-2 is an *in vitro* artifact and does not take place *in vivo*, or methylation of HPH-2 is induced by an unknown stimuli not present in normal cell culture conditions resulting in undetectable levels of methylated HPH-2 protein.

Transfection of PRMT1 and PRMT5 specific siRNA oligos caused significant reduction of each protein; however, different reporter cell lines responded differently (data not shown). In some cells, suppression of methyltransferases induced reporter expression similar to methyltransferase inhibition by AdOx, whereas in some other reporter cell lines they did not affect or even reduced the reporter expression. In contrast to suppression of a single PRMT enzyme by siRNA, AdOx inhibits PRMTs all together eliminating the issue of

redundancy between PRMT enzymes (type I (PRMT1, PRMT3, PRMT4, and PRMT6) and type II (PRMT5 and PRMT7)) (Bedford and Richard 2005).

Finally, we showed that under hypoxic conditions the general methyltransferase inhibitor AdOx further induces both the α-subunit stability (Fig. 14A) and transcriptional activity of HIF (Fig. 15A) in time- and dose-dependent manner. Retention of HIF-α protein at a higher level in AdOx treated cell compared to untreated cells at later time points of hypoxia (Fig. 14B) suggested that methylation might be involved in adaptation of HPH enzymes to low levels of oxygen and regain their hydroxylase activity. Consequences of arginine methylation vary immensely between proteins. For example; subcellular localization of Sam68 (a protein involved RNA processing), activity of transcriptional co-activator p300 and elongation factor SPT5, and assembly of ribosomal protein S2 into ribosome are known to be regulated by methylation (Bedford and Richard 2005). Comparable hydroxylase activity of R295A mutant (eliminating the primary methylation site) and wild-type enzyme (Fig. 13) indicates that methylation does not directly affect the hydroxylase activity. Perhaps, methylation alters subcellular localization of HPH-2 or interaction of HPH-2 with a regulatory protein *in vivo*. Similar nuclear and cytoplasmic distribution of HPH-2 under hypoxic and normoxic condition (Chapter 2, Fig. 5) favors the later possibility. One such HPH-2 interaction partner is OS-9, which enhances hydroxylation of HIF-α subunit by tethering it to HPH-2. Therefore, it needs to be determined whether the OS-9-HPH-2 interaction is regulated by methylation or not. Future studies addressing of how methylation of HPH enzymes occurs, if at all, will establish another layer of HPH regulation and thus deepen our understanding of the hypoxia response pathway.

CHAPTER FIVE
Conclusions and Future Directions

Oxygen homeostasis is of vital importance for cell survival. The cellular hypoxia response pathway, activated under hypoxic conditions, counteracts the reduction in oxygen availability by enhancing oxygen supply (i.e. angiogenesis and erythropoiesis) and reducing cellular oxygen demand (activation of glycolysis for oxygen-independent energy production) among other processes (Semenza 2000; Bruick 2003). Cellular hypoxia is encountered under a variety of circumstances either physiological or pathophysiological (Semenza 2000). A hypoxic environment may arise as a direct consequence of lower oxygen partial pressure at high altitudes or limited oxygen delivery due to underdeveloped vasculature at early stages of development. Similarly, a hypoxic cellular milieu may also be caused by disease conditions such as ischemia due to attenuation of blood/oxygen flow because of an occlusion in blood vessel, and cancer due to outgrowth of cancer cells to local vascularization in solid tumors (Semenza 2000). Cellular response to hypoxia is orchestrated by the master regulator transcription factor called Hypoxia Inducible Factor (HIF).

HIF is a dimeric transcription factor composed of oxygen sensitive α- and oxygen insensitive β-subunits (HIF-1β/ARNT) (Semenza 2001; Bruick 2003; Ozer and Bruick 2007). Under hypoxic conditions, the α-subunit is stabilized and HIF activates expression of its target genes by recruiting transcriptional co-activators upon binding to the Hypoxia Response Elements (HRE) located around the gene. The α-subunit is degraded via proteosome following ubiquitylation by pVHL-containing E3 ubiquitin ligase complex under

normal oxygen levels (normoxia). Likewise, transcriptional activity of HIF is sensitive to oxygen levels. Through these mechanisms, HIF is inactivated under normal oxygen levels and activated only under hypoxic conditions. Central to oxygen-dependent regulation of HIF are the oxygen-sensory HIF prolyl (HPH-1, -2, and -3) and asparaginyl (FIH-1) hydroxylases which promote degradation of α-subunit and inhibit transcriptional co-activator recruitment, respectively (Semenza 2001; Bruick 2003; Safran and Kaelin 2003; Ozer and Bruick 2007). In an effort to further our understanding of HIF regulation and in particular regulation of HIF hydroxylases, we studied protein-protein interactions of both HIF prolyl and asparaginyl hydroxylases.

HPH-2 interacting protein ING4 acts as a repressor of HIF transcriptional activity

We identified Inhibitor of Growth family member 4 (ING4) as a HPH-2 interacting protein in a yeast two-hybrid screen and later confirmed this interaction by various methods (Fig. 2.3) (Ozer, Wu et al. 2005). As opposed to its reported cytoplasmic localization as a GFP-fusion protein (Metzen, Berchner-Pfannschmidt et al. 2003), we showed that a substantial fraction of endogenous HPH-2 protein resides in the nucleus together with ING4 (Fig. 2.5), where the two proteins form a ternary complex with the HIF transcription factor at Hypoxia Response Elements (HRE)-containing promoters *in vivo* (Fig. 2.10) (Ozer, Wu et al. 2005). Further characterization of the ING4-HPH-2 interaction revealed an unexpected function of ING4, that is, repression of HIF transcriptional activity. Suppression of the ING4 protein promotes further induction of HIF target genes under hypoxic conditions (Fig. 2.9) and this effect is dependent on chromatin structure (Fig. 2.11) (Ozer, Wu et al. 2005).

Based on our data we proposed a model whereby ING4 is recruited by HIF-associated HPH-2 to modulate its transcriptional activity (Fig. 5.1) (Ozer and Bruick 2005). Such a model has two major implications for regulation of HIF transcription factor. First, a constitutive interaction between HPH-2 and HIF α-subunit under hypoxia allows HPH-2 to target HIF-α for degradation as soon as oxygen becomes available explaining the short half-life of HIF-α upon reoxygenation (Wang, Jiang et al. 1995; Jewell, Kvietikova et al. 2001). Second, it indicates a novel function of HPH-2, that is, repression of HIF transcriptional activity under hypoxia, in addition to its primary function, targeting HIF-α for degradation under normoxia. A similar HIF co-repressor function of HPH-2 has been demonstrated by Eric Huang and his colleagues (To and Huang 2005). Different from our model, they propose that HPH-2 represses HIF transcriptional activity by binding to the ODD/NTAD region of HIF-α and thus preventing association of HIF-α NTAD with co-activators; however, we believe that recruitment of ING4 and perhaps additional proteins is responsible, at least in part, for the repression of HIF transcriptional activity. This transcriptional repression by HPH-2 is not the major mode of HIF regulation, but instead serves as a fine-tuning mechanism that determines the absolute HIF activity (Ozer and Bruick 2005). Even then, misregulation of HIF activity is likely to contribute to angiogenesis and progression of brain tumors where ING4 expression level is reduced (Garkavtsev, Kozin et al. 2004). Likewise, (mis)regulation of HIF target genes may also participate in other processes where ING4 had been implicated such as apoptosis, cell proliferation (Shiseki, Nagashima et al. 2003), cell cycle control (Zhang, Xu et al. 2004), and contact inhibition (Kim, Chin et al. 2004). Based

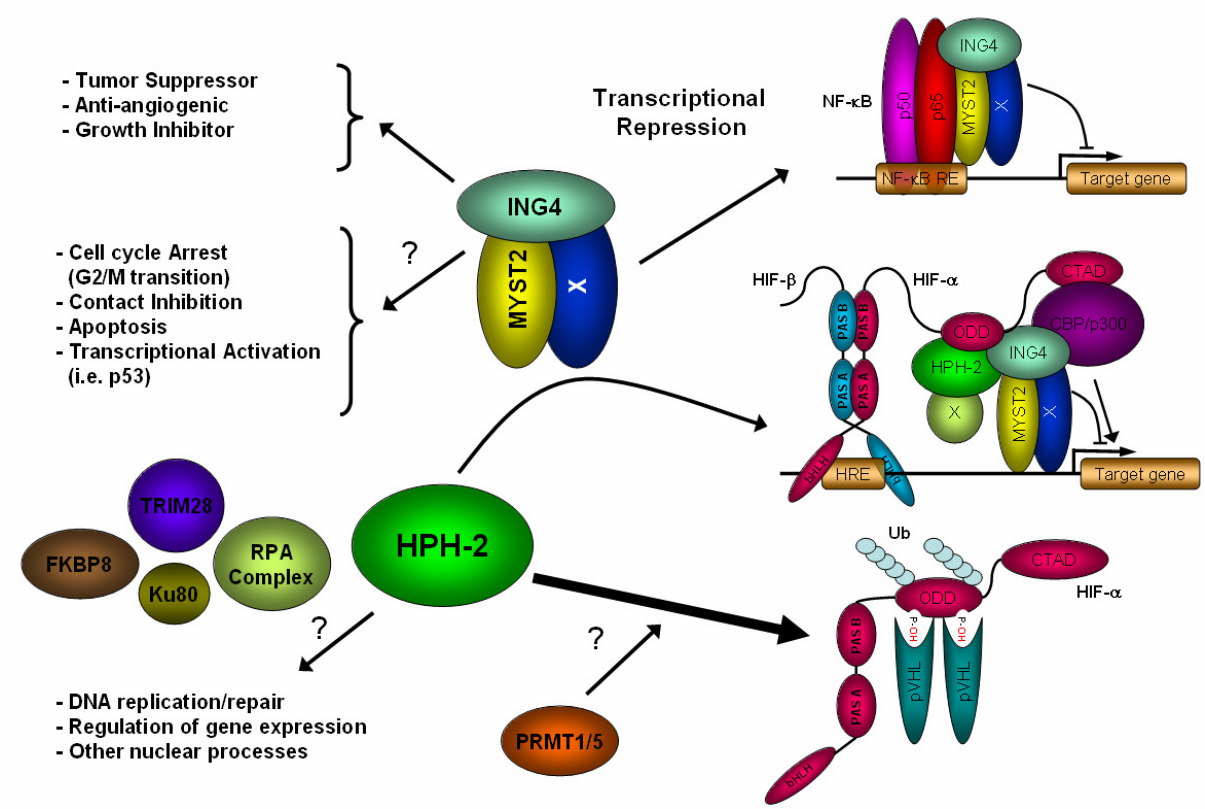

Figure 5.1 Proposed model for the function of HPH-2 interacting proteins
HPH-2 targets HIF α-subunit to ubiquitin-mediated proteosomal degradation by hydroxylating proline residues in the Oxygen-dependent Degradation Domain (ODD). In addition, HPH-2 inhibits HIF transcriptional activity by recruiting ING4-MYST2 complex. Similar ING4-MYST2 complex also targets NF-κB transcription factor and represses its activity. (Mis)Regulation of these transcription factors and their targets provide a link between ING4 and cellular processes that ING4 have been implicated in. Novel protein-protein interactions of HPH-2 described in this thesis suggest a role for this enzyme in pathways besides hypoxia response and an alternative mode of regulation for HPH-2 by post-translational modification (methylation by PRMT enzymes).

on its interaction with ING4, one might also speculate that HPH-2 may also play a role in these processes.

Interaction between ING4 and HPH-2 is mediated through regions that are highly similar between the members of Inhibitor of Growth (ING) and HIF Prolyl Hydroxylase protein families, the Plant Homeodomain (PHD) and the catalytic prolyl hydroxylase domain, respectively. Therefore, we suspect that other ING family proteins may also interact with HPH-2 enzyme and function in HIF regulation. We are confident of the HIF regulatory functions of ING4 protein; however, it is not clear whether its effect is exclusively mediated through HPH-2 or whether other HPH enzymes are involved. Futhermore, other ING proteins may also interact with HPH-1 and HPH-3, and these potential protein-protein interactions between ING and HPH family proteins deserve further studies and may add an additional layer of complexity to the regulation of hypoxia response pathway.

Regulation of HPH-2-ING4 interaction has not been investigated yet. To this end, subcellular localization/nuclear translocation, post-translational modification(s) and other binding partners of each protein need to be studied. In a recent proteomics screen, ING4 was found to be acetylated at three different lysine residues within the nuclear localization signal (Kim, Sprung et al. 2006); however, the functional consequences of these post-translational modifications are unknown. To date, no post-translational modification of HPH-2 protein, except the potential arginine methylation (discussed below), has been reported. Furthermore, ING4-HPH-2 interaction may also be affected by binding of cellular metabolites to their corresponding interaction domains. PHD of ING family proteins were shown to bind certain phosphoinositides (Gozani, Karuman et al. 2003) and recognize trimethylated lysine residues

of various histones (Palacios, Garcia et al. 2006; Pena, Davrazou et al. 2006). Such regulatory mechanisms may ultimately provide a link between various signaling pathways and regulation of HIF or other processes in which ING4 and HPH-2 are involved.

Similar to its effects on HIF, we (Chapter 3) and others (Garkavtsev, Kozin et al. 2004) have shown that ING4 also represses the activity of yet another hypoxia inducible transcription factor, NF-κB. Contrary to its repressive effects, ING4 was found to activate p53 transcription factor by inducing its acetylation (Shiseki, Nagashima et al. 2003). All together, regulation these and perhaps other transcription factors determines the role of ING4 in a given process such as apoptosis, cell proliferation, and cell cycle arrest. Microarray analysis of gene expression with cells overexpressing ING4 and ING4 suppressed may help identification of additional ING4-regulated transcription factors and target genes that connect ING4 to the aforementioned processes.

ING4 forms a complex with MYST2 and JADE3 to repress HIF and NF-κB activity

In order to shed some light on the underlying mechanism of transcriptional repression by ING4, we purified ING4 using the Tandem Affinity Purification (TAP) method. To our surprise, ING4 co-purified with the MYST2/HBO1 histone acetyltransferase and an uncharacterized PHD-containing protein JADE3 instead of a histone deacetylase complex, which one would have predicted based on the repressive effect of ING4 (Fig. 3.7 and Table 3.3). A similar ING4 protein complex has been purified by others as well (Doyon, Cayrou et al. 2006). This complex has been studied with respect to MYST2's histone acetyltransferase activity but not with respect to its role in transcriptional regulation (Doyon, Cayrou et al.

2006). MYST2 has been shown to activate the progesterone receptor as expected from a histone acetyltransferase (Georgiakaki, Chabbert-Buffet et al. 2006); however, the yeast homologs of MYST2, SAS2 (Ehrenhofer-Murray, Rivier et al. 1997) and SAS3 (Reifsnyder, Lowell et al. 1996), and recently MYST2 itself, were shown to function as transcriptional repressors (Sharma, Zarnegar et al. 2000). These contradictory effects of MYST2 are consistent with the dual nature of ING4 function, an activator of p53 and a repressor of HIF and NF-κB. What governs the distinction between the transcription factors to be induced and the ones to be repressed is unknown.

The actual mechanism of transcriptional repression by ING4-MYST2 complex also requires further investigation. The histone acetyltransferase (HAT) domain of MYST2 was shown not to be required for NF-κB repression for which the proposed mechanism of MYST2 function was the sequestration of a required component away from the transcription factor (Contzler, Regamey et al. 2006). Our in vivo data contradict such a model, instead suggest a model whereby ING4 and MYST2 are associated with HIF and NF-κB and actively involved in repression of transcription (Fig. 5.1). However, we do not know whether ING4-MYST2 complex recruits transcriptional co-repressor proteins/protein complexes to do the job or it displaces a more potent co-activator thereby resulting in a relative repression. BRMS1, a component of the mSIN3A-HDAC co-repressor complex (Meehan, Samant et al. 2004), identified as an ING4 interacting protein in yeast two-hybrid screen represents a good candidate for the former mechanism. In addition, several lines of evidence implicate BRMS1 in regulation of HIF and NF-κB. Proteins shown to interact with the mSIN3A complex (histone deacetylases (HDAC1 and HDAC2) (Meehan, Samant et al. 2004) and components

of SWI/SNF chromatin remodeling complex (Sif, Saurin et al. 2001)) were found to alter activity of HIF (Mahon, Hirota et al. 2001; Wang, Zhang et al. 2004), and recently BRMS1 has been shown to target NF-κB (Cicek, Fukuyama et al. 2005; Liu, Smith et al. 2006; Samant, Clark et al. 2007). For the latter mechanism, both HIF and NF-κB are known to interact with numerous transcriptional co-activators including CBP/p300 and SRC-1 (Hoffmann, Natoli et al. 2006) and a competition between these co-activators and the ING4-MYST2 HAT complex for HIF and NF-κB binding may be the underlying mechanism of their repression. Such a model may also provide an explanation for the transcriptional activator function of ING4-MYST2 complex as it would normally be expected of an HAT complex, given that there is no competition with other co-activators.

In addition, it remains possible that HAT activity might be required for repression of HIF, in which case, the target of acetylation (either the transcription factor itself or a component of the transcriptional machinery) needs to be identified.

Identification of proteins associated with HIF hydroxylases & Regulation of HIF Prolyl Hydroxylase activity by methylation

Recent data suggest that in addition to their oxygen sensory role in regulation of HIF, HIF prolyl (HPH-1, -2, -3) and asparaginyl (FIH-1) hydroxylases also function as sensors of cellular metabolites and reactive oxygen species (Lu, Dalgard et al. 2005; Selak, Armour et al. 2005) that not only regulates HIF pathway but also other pathways (Cummins, Berra et al. 2006). We purified HPH-1, HPH-2, HPH-3, and FIH-1 proteins using the tandem affinity purification (TAP) method in order to identify HPH and FIH-1 associated proteins. Our

expectation was that some of these proteins could be i) novel substrates of these enzymes linking oxygen levels, oxidative stress and metabolic state of cell to signaling pathways other than HIF, ii) regulators of these enzymes that either by direct protein-protein interaction or by post-translational modifications alter HIF hydroxylase activities, or iii) scaffolding proteins that recruit these enzymes to modulate other cellular processes..

HPH-1, consistent with the results of an earlier published work (Masson, Appelhoff et al. 2004), co-purified with subunits of the TRiC chaperonin complex (Table 5.1). Although it is possible that TRiC chaperonin complex is involved in folding of HPH-1 as proposed in that previous work, it is also possible that HPH-1 may be a component of this complex. Within this complex HPH-1 might serve two functions. First, it may hydroxylate and target proteins which cannot be properly folded to proteosomal degradation similar to HIF, or it may indeed aid in protein folding. Interestingly, in *Dictyostelium* (more commonly known as slime mould) prolyl hydroxylation of SKP1 (Teng-umnuay, Morris et al. 1998), a component of SCF E3-ubiquitin ligase complex (Zheng, Schulman et al. 2002), is required for its assembly into a functional complex (Cope and Deshaies 2003). The enzyme responsible for SKP1 prolyl hydroxylation was recently identified and found to be similar to HIF prolyl hydroxylases in protein sequence and in target specificity (the target proline of SKP1 resides within a LXXLAP motif similar to hydroxylation sites of HIF α-subunits). Further analysis is required to delineate such a function of HPH-1 in mammals. HPH-1 knockout mice or cells derived from these animals will be a valuable tool to investigate this potential function of the HPH-1 enzyme.

Table 5.1 Known and novel interactions of HIF prolyl and asparaginyl hydroxylase enzymes

Enzymes	Interacting Proteins	Function/Effect	References
HPH-1	HIF α-subunits	HPH substrates	(Bruick and McKnight 2001) (Epstein, Gleadle et al. 2001) (Ivan, Haberberger et al. 2002)
	TRiC chaperonin complex	Potential regulator of HPH-1 folding	(Masson, Appelhoff et al. 2004) (Ozer and Bruick UpD)
	SIAH1/2	Promotes HPH-1 degradation	(Nakayama, Frew et al. 2004)
	OS-9	Activates HIF prolyl hydroxylase activity	(Baek, Mahon et al. 2005)
HPH-2	HIF α-subunits	HPH substrates	(Bruick and McKnight 2001) (Epstein, Gleadle et al. 2001) (Ivan, Haberberger et al. 2002)
		Repression of HIF transcriptional activity	(Ozer, Wu et al. 2005) (To and Huang 2005)
	ING4	Regulates HIF transcriptional activity together with HPH-2	(Ozer, Wu et al. 2005)
	SIAH2	Unknown	(Nakayama, Frew et al. 2004)
	OS-9	Activates HIF prolyl hydroxylase activity	(Baek, Mahon et al. 2005)
	MOV10	Unknown	(Ozer and Bruick UpD)
	TRIM28	Unknown	(Ozer and Bruick UpD)
	Ku80	Unknown	(Ozer and Bruick UpD)
	DDX50	Unknown	(Ozer and Bruick UpD)
	FKBP8	Destabilizes HPH-2	(Barth, Nesper et al. 2007) (Ozer and Bruick UpD)
	PRMT1	Methylates HPH enzymes, potential activator of HPH enzymes	(Ozer and Bruick UpD)
	PRMT5	Unknown	(Ozer and Bruick UpD)
	IGF2BP1	Unknown	(Ozer and Bruick UpD)
	ILF3	Unknown	(Ozer and Bruick UpD)
	RPA1/2/3	Unknown	(Ozer and Bruick UpD)
HPH-3	HIF α-subunits	HPH substrates	(Bruick and McKnight 2001) (Epstein, Gleadle et al. 2001) (Ivan, Haberberger et al. 2002)
	SIAH1/2	Promotes HPH-3 degradation	(Nakayama, Frew et al. 2004)
	MORG1	Molecular scaffold for HPH-3	(Hopfer, Hopfer et al. 2006)
FIH-1	HIF α-subunits	FIH-1 substrates	(Mahon, Hirota et al. 2001) (Hewitson, McNeill et al. 2002) (Lando, Peet et al. 2002)
	p105 (NFκB1)	FIH-1 substrates	(Cockman, Lancaster et al. 2006)
	IκBα	FIH-1 substrates	(Cockman, Lancaster et al. 2006)
	pVHL	Inhibits HIF transcriptional activity together with FIH-1	(Mahon, Hirota et al. 2001)
	HDAC1/3	Inhibit HIF transcriptional activity together with FIH-1	(Mahon, Hirota et al. 2001)
	SIAH1	Promotes FIH-1 degradation	(Fukuba, Yamashita et al. 2007)

UpD: Unpublished data

Our TAP purification of HPH-3 and FIH-1 did not result in identification of any novel interactions of these proteins (Table 5.1). Purification of HPH-2, on the other hand, yielded several HPH-2 associated proteins, some of which are known nuclear proteins (Table 5.1). Sequence analysis of these proteins revealed potential hydroxylation motifs similar to LXXLAP motif found on HIF α-subunits; however, none of these proteins served as substrates of HPH-2 enzyme *in vitro* (Figs. 4.7 and 4.8) or *in vivo* (Fig. 4.6D). Furthermore, these proteins did not affect hydroxylase activity of HPH-2 *in vitro* (Figs. 4.6A and 4.6B) or endogenous HPH enzymes *in vivo* (Fig. 4.6C). We then focused our attention on two protein arginine methyltransferases, PRMT5 and PRMT1, identified as HPH-2 interacting proteins by tandem affinity purification (Table 4.3) and yeast two-hybrid screening (Chapter 1), respectively.

Protein arginine methyltransferase (PRMT) enzymes utilize S-adenosyl-L-methinone (SAM) co-factor as the methyl- group donor (Bedford and Richard 2005). Speculating that potential methylation-mediated effects of these proteins on HPH-2 could have been masked due to absence or insufficient levels of SAM in *in vitro* (Figs. 4.6A and 4.6B) and *in vivo* assays (Fig. 4.6C), we tested methylation of HPH-2 by PRMT1 and PRMT5. Indeed, PRMT1 was able to methylate HPH-2 and HPH-3, but not FIH-1, establishing the specificity of PRMT1 against HIF prolyl hydroxylases (Fig. 4.10). Inefficiency of methylation (around ~3%) prevented us to study its effects on HIF prolyl hydroxylase activity *in vitro*; however, treatment of cells with a general methyltransferase inhibitor, AdOx, resulted in further stabilization of HIF-1α under hypoxic conditions (Figs. 4.13 and 4.14) suggesting a HPH activating effect of methylation (Fig. 5.1). Further study is required to establish that the effect

of AdOx treatment is a direct consequence of inhibition of HPH methylation but not a non-specific artifact.

Activity of HPH-2 was not affected *in vitro* by mutations at the primary methylation sites Arg295 (Fig. 4.12) or Lys297 (data not shown), which suggests that methylation does not directly alter the activity of enzyme but instead regulates it indirectly perhaps by modulating a critical interaction of HPH enzymes. To this end, protein-protein interactions of HPH enzymes, especially HPH-2-OS-9 which was shown to enhance prolyl hydroxylation of HIF-α by tethering HPH-2 enzyme to HIF-α substrate (Baek, Mahon et al. 2005), need to be reevaluated with regards to the methylation status of HPH-2. Furthermore, to our knowledge, methylation is the first post-translational modification identified for HPH enzymes, which may provide a novel link between various signaling pathways or cellular metabolic state (i.e. SAM level) and HIF prolyl hydroxylases activity/HIF pathway.

Furthermore, it is tempting to speculate that HPH-2 is involved in different cellular processes such as DNA replication and repair (Fig. 5.1), besides the hypoxia response pathway. Interactions of HPH-2 with proteins implicated in DNA replication (i.e. Ku80 (Matheos, Ruiz et al. 2002; Sibani, Price et al. 2005), RPA1, RPA2 (Fanning, Klimovich et al. 2006), and RuvB-like 2 (Kanemaki, Kurokawa et al. 1999)) (Table 4.3) are in agreement with this idea. In yeast, DNA replication was shown to be coordinated with the metabolic/oxidative cycle (Tu, Kudlicki et al. 2005). By analogy, one would predict that regulation of DNA replication would be interconnected with the metabolic state of mammalian cells as well. Based on their sensitivity to cellular metabolites as well as levels of oxygen and reactive oxygen species, HPH enzymes are perfect candidates for such an

interconnection. However, the exact function of HPH-2 in DNA replication/repair and other cellular processes require a detailed future study.

A final overview of hypoxia response pathway: An update on HIF hydroxylases

The hypoxia response pathway, in particular, regulation of HIF by HIF prolyl and asparaginyl hydroxylases is an active research field in which many discoveries have been made over the last five years. During this time, HIF hydroxylases, discovered as the oxygen sensors of the hypoxia response pathway, have been recognized as major regulatory enzymes. With recent studies, it is becoming more and more evident that these enzymes are not just simple oxygen sensors as once thought, instead they act as central decision makers which integrate various signals and formulate a response via HIF and perhaps other factors such as NF-κB (Cummins, Berra et al. 2006)). Signals that mediate HIF hydroxylase activity include oxygen (Bruick and McKnight 2001; Epstein, Gleadle et al. 2001; Ivan, Haberberger et al. 2002), cellular metabolites (Lu, Dalgard et al. 2005; Selak, Armour et al. 2005), and reactive oxygen species (Gerald, Berra et al. 2004) (See Chapter 1 for details).

Furthermore, activities of HIF hydroxylases are controlled by a complex set of regulatory mechanisms including autoinhibition (Choi, Lee et al. 2005), enzyme-substrate interaction (Baek, Mahon et al. 2005), and expression level (Appelhoff, Tian et al. 2004; Khanna, Roy et al. 2006; Stiehl, Wirthner et al. 2006). Expression of HPH-1 and HPH-2 are induced by HIF under hypoxic conditions constituting a negative feedback loop for regulation of HIF stability (Appelhoff, Tian et al. 2004; Khanna, Roy et al. 2006; Stiehl, Wirthner et al. 2006). In addition, the stability of HIF hydroxylases are actively regulated

(Nakayama, Frew et al. 2004; Barth, Nesper et al. 2007; Fukuba, Yamashita et al. 2007). FKBP38, a peptidyl prolyl isomerase, destabilizes the HPH-2 protein (Barth, Nesper et al. 2007). Likewise, SIAH1/2 proteins, components of E3 ubiquitin ligase complexes were shown to destabilize HPH-1 and HPH-3 (Nakayama, Frew et al. 2004), and FIH-1 (Fukuba, Yamashita et al. 2007). Induction of SIAH expression by hypoxia constitutes a positive feedback loop with SIAH-HPH-HIF proteins as opposed to negative feedback loop formed by HPH and HIF. All together, these numerous regulatory mechanism (and yet unidentified ones) are a good indication of how fundamentally important the HIF hydroxylases are.

Misregulation of HIF has long been associated with disease conditions. Overexpression of HIF in cancer cells and its preventive effects in ischemic brain and heart injuries made HIF a popular target for therapy (Paul, Simons et al. 2004; Belozerov and Van Meir 2005; Semenza 2006). HPH enzymes have gained popularity as a therapeutic target as well, since alterations in HPH activity are likely to impact HIF pathway dramatically. Indeed, strategies targeting HPH enzymes showed promising results in prevention of ischemic brain (Siddiq, Ayoub et al. 2005), heart (Ockaili, Natarajan et al. 2005; Natarajan, Salloum et al. 2006), and muscle injuries (Milkiewicz, Pugh et al. 2004).

We made two significant contributions to the current understanding of hypoxia response pathway centered around the HIF transcription factor and HIF modifying prolyl and asparaginyl hydroxylases that are of biological and medical importance. First, we (Ozer, Wu et al. 2005) and others (To and Huang 2005) identified a novel function of HPH-2 enzyme, that is, repression of HIF transcriptional activity. We also showed that recruitment of ING4 protein by HPH-2 to HIF is at least one of the underlying mechanisms of this repression

under hypoxia (Chapter 2). Second, we identified a set of novel protein-protein interactions made by HPH-2 and a potential regulatory post-translational modification. Several signaling pathways have been implicated in regulation of hypoxia response pathway. Overexpression of v-Src and RasV12 oncogenes induce HIF α-subunit stabilization even under normoxic conditions by inhibiting its prolyl hydroxylation (Chan, Sutphin et al. 2002). Proteins involved in other signaling pathways (i.e. MAPK, AKT, mTOR (Bardos and Ashcroft 2004)) are long known to affect HIF α-subunit protein levels some of which might do so by altering HPH activity instead of HIF α-subunit expression. It is likely that HPH enzymes are at the crossroad of many other signaling pathways as well; however, no post-tranlational modification of the HPH enzymes has been reported yet. We showed that HPH enzymes can be methylated by PRMT1 *in vitro* and our in vivo data suggest that methylation activates the HPH prolyl hydroxylase activity. Finally, we believe that the novel protein-protein interactions and the methylation of HPH enzymes will likely provide a link between input and output effector functions of HPH enzymes and the future studies outlined will be of great importance not only for a more complete understanding of the cellular response to changes in oxygen concentration but also changes in cellular environment.

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VITAE

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