

**INVESTIGATING POTENTIAL POST-
TRANSLATIONAL MODIFICATION OF
FACTOR-INHIBITING HIF (FIH-1)**

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TRANSLATIONAL MODIFICATION OF
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ABSTRACT

The Hypoxia Inducible Factors (HIFs) are widely expressed transcription factors critical for altering gene expression in hypoxic cells and enabling cellular adaptation to conditions of limited oxygen availability. The HIFs are labile and inactive when oxygen levels are sufficient to meet cellular oxygen demand, but become stabilised and transcriptionally active when oxygen levels decrease. Factor Inhibiting HIF-1 α (FIH-1) is an asparaginyl hydroxylase that was first identified via its interaction with the HIF- α subunit. The canonical role for the enzyme involves the oxygen-dependent regulation of HIF transcriptional activity. In normoxia, FIH-1 hydroxylates an asparaginyl residue in the C-terminal transactivation domain of HIF- α , blocking interaction with vital transcriptional coactivators and abrogating HIF transcriptional activity. As FIH-1 requires oxygen for hydroxylation, activity of the enzyme decreases with decreasing oxygen levels, allowing HIF- α to escape hydroxylation and consequently activate target gene expression during periods of insufficient oxygen tension.

More recently, FIH-1 has been found to bind and hydroxylate a number of proteins containing ankyrin repeat domains (ARDs). However, despite the prevalence of ARD hydroxylation, there is, as yet, no established role attributed to these modification events. Additionally, FIH-1 knockout mice have revealed a surprising role for FIH-1 as a neuronal regulator of metabolism, suggesting a novel, cell-specific role for the enzyme.

As FIH-1 requires O₂ for catalysis, the availability of intracellular oxygen is thought to determine activity of the enzyme, branding FIH-1 as a putative cellular oxygen sensor. Aside from modulation of enzyme activity by oxygen levels, little is known about the regulation of FIH-1. Several lines of evidence have suggested that FIH-1 exhibits cell type-specific differences in activity toward HIF- α substrates that may act in addition to or separately from the regulation of enzyme activity by levels of available oxygen.

The primary aim of this work was to investigate the post-translational modification (PTM) of FIH-1 in order to uncover any additional regulatory mechanisms that may exist. Two-dimensional electrophoresis (2-DE) experiments from a number of cancer cell lines and mouse embryonic fibroblasts revealed heterogeneity in the isoelectric point of FIH-1, suggesting the existence of multiple post-translationally-modified forms of the enzyme. Overexpressed FIH-1 was affinity purified for mass spectrometric (MS) identification of PTMs. MS analysis was able to demonstrate asparaginyl deamidation and methionine oxidation. It is unclear, however, whether these modifications represent modifications occurring in the cellular environment or during sample processing.

Due to inefficient purification of FIH-1 from cells, it could not be ascertained if phosphorylation was present. However, phosphatase treatment of cell lysate followed by 2-DE consistently showed a decrease in spot number and shift of FIH-1 spots to a more basic position on a 2-D field, suggesting that the isoelectric point differences of FIH-1 could be attributed, in part, to phosphorylation. Furthermore, *in vitro* phosphorylation assays indicated that recombinant FIH-1 was able to be phosphorylated by kinases supplied by cell lysate.

In summary, the work presented here provides evidence for the existence of novel PTMs of FIH-1, and suggests that FIH-1 may be a kinase substrate.

CANDIDATES DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Karolina Lisy and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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ABBREVIATIONS

2-DE	Two-Dimensional Electrophoresis
2-OG	2-oxoglutarate
3'UTR	3' untranslated region
6His	6x Histidine
A ₂₈₀	Absorbance at 280 nm
Ank	Ankyrin
Amp	Ampicillin
ARD	Ankyrin repeat domain
Arnt	Aryl hydrocarbon nuclear translocator
APS	Ammonium persulphate
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
BME	Beta mercaptoethanol
bp	base pairs
BSA	Bovine serum albumin
CAD	Carboxy-terminal transactivation domain
CA9	Carbonic Anhydrase 9
CBP	CREB-binding protein
CCRCC	Clear cell renal cell carcinoma
CD	Deamidation coefficient
CDP	CCAAT-displacement protein
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CNBr	Cyanogen Bromide
DAPI	4',6-diamidino-2-phenylindole

DBD	DNA binding domain
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DP	2,2' dipyridyl
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoetin
EtBr	Ethidium bromide
FCS	Fetal calf serum
FIH-1	Factor Inhibiting HIF
GFP	Green fluorescent protein
Glut	Glucose transporter
GSV	Glut4 storage vesicle
GTS	Glycine/trizma base/SDS
HDAC	Histone deacetylase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIF- α	Hypoxia inducible factor α subunit
HIF- β	Hypoxia inducible factor β subunit
HIF	Hypoxia inducible factor (heterodimer)
hnRNP B1	Heterogeneous nuclear ribonucleoprotein B1
HNSCC	Head and neck squamous cell carcinoma
HRE	Hypoxic response element
HRP	Horseradish peroxidase
ICD	Intracellular domain
IEF	Isoelectric focusing

IF	Immunofluorescence
Ig	Immunoglobulin
ILK-1	Integrin-linked kinase 1
IMAC	Immobilised metal affinity chromatography
IP	Immunoprecipitation
IPG	Immobilised pH gradient
IPTG	Isopropyl- β -D-thiogalactopyranoside
IRAP	Insulin-regulated aminopeptidase
IRES	Internal ribosome entry site
Kb	Kilobase
KDa	Kilodalton
K_m	Michaelis constant
Ko	knockout
LB	Luria Broth
LDHA	Lactate dehydrogenase A
Luc	Luciferase
MALDI	Matrix-assisted laser desorption/ionisation
MAPK	Mitogen-activated protein kinase
MBP	Maltose binding protein
MEF	Mouse Embryonic Fibroblast
miRNA	Micro RNA
MQ	Milli-Q
mRNA	Messenger ribonucleic acid
MS	Mass Spectrometry
MT1-MMP	Membrane type 1 matrix metalloprotease
MW	Molecular weight
NAD	N-terminal transactivation domain
Ni-IDA	Nickel nitrilotriacetic acid

NO	Nitric oxide
OD ₆₀₀	Optical density at 600 nm
ODDD	Oxygen-Dependent Degradation Domain
O/N	Overnight
PAGE	Polyacrylamide gel electrophoresis
PAS	Per-Arnt-SIM homology domain
PBS	Phosphate buffered saline
PBT	Phosphate buffered saline with 0.1% Tween-20
PCR	Polymerase chain reaction
PI3K	phosphatidylinositol 3 kinase
Pen/Strep	Penicillin/streptomycin
PGK1	Phosphoglycerate kinase 1
PHD	Prolyl hydroxylase domain-containing protein
pI	Isoelectric point
PKC ζ	Protein kinase C zeta
PM	Plasma membrane
PMSF	Phenylmethyl sulfonyl fluoride
PoAb	Polyclonal antibody
PP1R12C	Protein phosphatase 1 regulatory subunit 12 C
PPAse	Phosphatase
PTM	Post-translational modification
pVHL	Von Hippel Lindau protein
RCC	Renal cell carcinoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SB3-10	N-decyl-N,N-dimethyl-3-ammonio-l-propane-sulfonate
SDS	Sodium dodecyl sulfate

SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	Small interfering RNA
TAD	Transactivation domain
TCA cycle	Tricarboxylic acid cycle
TE	Tris/EDTA
TEMED	N,N,N ¹ ,N ¹ -teramethyl-ethylenediamide
Tween-20	polyoxyethylene-sorbitan monolaurate
Tris	Tris (hydroxymethyl) aminomethane
Trx	Thioredoxin
VEGF	Vascular endothelial growth factor
VHr	Volt hour
V _{max}	Maximum rate
WB	Western blot
WCE	Whole cell extract
WCEB	Whole cell extract buffer
Wt	Wildtype
Y2H	Yeast 2 hybrid

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