# Substrate Specificity of Factor Inhibiting HIF-1 (FIH-1)

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# Thesis Abstract

To detect and respond to the detrimental situation of hypoxia, metazoan cells employ $O_2$-sensing prolyl and asparaginyl hydroxylases which directly utilise $O_2$ to hydroxylate and regulate the Hypoxia Inducible transcription Factor-α (HIF-α). This thesis focuses upon the asparaginyl hydroxylase, 'Factor Inhibiting HIF-1 (FIH-1), which represses HIF-α in normoxia by asparaginyl hydroxylation of its C-terminal *trans*-Activation Domain (CAD). During hypoxia FIH-1 is inhibited, allowing non-hydroxylated HIF-α to drive expression of over 70 target genes, leading to tissue and cellular changes that increase $O_2$ supply and reduce its consumption. This response is central to normal physiology and to the pathophysiology of diseases, including stroke and cancer. The pivotal role of FIH-1 in regulating these processes invites its characterisation, as a key cellular $O_2$-sensor and therapeutic target. This thesis contributes important information by elucidating a novel FIH-1 substrate and by defining numerous FIH-1 substrate recognition determinants.

The first aim was to investigate the cell-fate regulator Notch1 as a potential FIH-1 substrate, due to myriad reports of Notch/hypoxic crosstalk and the discovery by collaborators that FIH-1 represses Notch1 activity. Mutagenesis, hydroxylation assays, affinity-purification and mass spectrometry techniques enabled definition of two asparaginyl hydroxylations of mouse Notch 1 ankyrin repeat domain (N1945 and N2012), performed by FIH-1 *in vitro*. These residues were likewise detected to be hydroxylated in mNotch1 expressed in mammalian cells. FIH-1 kinetic assays comparing mNotch1 ankyrin domain with the unstructured hHIF-1α CAD uncovered major distinctions between substrates; mNotch1 facilitated a 7-fold lower rate of cosubstrate turnover by FIH-1, but affinity was robust (>10-fold higher). Interrogation of the structure/affinity correlate implies FIH-1 binds unstable ankyrins preferentially. Functionally, a non-catalytic mechanism of Notch1 repression by FIH-1 is supported.

The second aim derived from literature analyses implicating threonine and RLL motifs in HIF-α as critical hydroxylation determinants. T796 (hHIF-1α) contacts FIH-1 and is a likely phospho-acceptor, thus a mimetic T796D mutant was generated and its hydroxylation kinetics compared with wildtype hHIF-1α CAD. *In vitro*, the mutant exhibited a 6-fold greater apparent $K_m$, explaining its constitutive activity in cell-based reporter assays, whereas wildtype hHIF-1α CAD is hydroxylated and thus repressed in normoxia by FIH-1. This indicates that phosphorylation reduces hydroxylation by FIH-1 *in vitro* and *in vivo*.

The RLL motif does not contact FIH-1 *in vitro* however RLL-AAA mutant HIF-α proteins are constitutively active in normoxia, suggesting resilience to hydroxylation within cells. To reconcile these data I predicted that a cellular Factor X functionalises the RLL motif as an FIH-1 binding site. Reporter assays, *in vitro* kinetic assays and interaction assays +/- lysate confirmed this hypothesis and additionally showed the motif to increase HIF-α protein turnover 8-fold. Numerous mechanisms for Factor X including nuclear export, post-translational modifications of FIH-1 or HIF-α, and involvement of small molecules, were experimentally examined, but deemed unlikely. Rather, the data imply Factor X to be a proteinaceous facilitator of a HIF-α/FIH-1 complex, thus proteomic capture screens are underway.

This research provides novel insight into FIH-1; its role in Notch/hypoxic crosstalk, its substrate recognition requirements, and its potential functions in cellular $O_2$-sensing.

# Candidates Declaration

To the best of my knowledge, 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 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.

I give consent to this copy of my thesis being made available in the University Library.

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21st December, 2007

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[#]These authors contributed equally to the work and should be considered equal first authors.

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# Table of Contents