Identification and Characterisation of Novel Substrates and Binding Partners of the Asparaginyl Hydroxylase, FIH

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B. Sc. (Hons)

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Abstract

The ability of cells to sense and respond to sub-optimal levels of oxygen is a key requirement for organism survival. Many cellular and physiological signalling pathways have been identified as being sensitive to oxygen levels, although remarkably, few of these pathways have been successfully linked with a genuine "oxygen sensing" molecule. Discovery of the 2-oxoglutarate-dependent asparaginyl hydroxylase, Factor Inhibiting HIF (FIH), as an oxygen sensitive regulator of the Hypoxia-inducible Factor (HIF) transcription factors has therefore led to considerable interest in the enzyme as a potential regulator of multiple oxygen-regulated processes. In this work, the known substrate repertoire of FIH was expanded using both yeast 2-hybrid (Y2H) and bioinformatics-based approaches. Potential positives identified in the Y2H screen included a number of proteins which contain an ankyrin repeat structural domain (ARD), and subsequent characterisation of these proteins by *in vitro* hydroxylation assay suggest that both Fetal Globin Inducing Factor (FGIF) and Serine/threonine-protein phosphatase 6 regulatory ankyrin repeat subunit B (PP6-ARS-B) are both novel substrates of FIH. Concomitant with this discovery, a number of other ARD-containing proteins have been reported as substrates of FIH in the literature, thus suggesting that hydroxylation of ARDs by FIH is common. Comparison of the target sites in these substrates reveals an "FIH preferred sequence" of LXXXXX[-]ϕN, however, it was discovered that FIH can also bind an ARD constrained in its folded state, suggesting that the tertiary fold of ankyrins may also participate in enzyme recruitment. Thus far, hydroxylation of ARDs reported in the literature has not been found to have a significant functional effect on ARD biology. Largely consistent with this, an assessment of the influence of FIH-mediated hydroxylation on IκBα stability and interaction with NFκB in this work suggested that the modification has only subtle effects. Furthermore, FIH was found to have no clear effect on the methyltransferase activity of the novel ARD-containing substrate, G9a. In addition to ARD-containing proteins, the Y2H also identified a number of non-ARD-containing proteins which displayed weak interactions with FIH that were inducible by the FIH inhibitor, DMOG. *In vitro* hydroxylation assays suggest that these proteins are not FIH substrates, and further study will be required to establish the biological significance of these interactions. Overall, this work suggests that FIH interfaces with many partners, and it remains to be determined how these interactions influence the function of FIH, as well as that of its substrates and binding proteins.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Rachel Hampton-Smith

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Acknowledgements

Since this is the part of the thesis that EVERYONE reads, I'd better do a decent job (and since I'm writing it at 1 in the afternoon, instead of at 3 in the morning (like my honours thesis), hopefully it will be a little more comprehensible).

It's customary to thank one's supervisor first, and I see no reason to break with that tradition. Dan, to use an American expression, I "lucked out" when I rather randomly selected your lab to do honours. You were friendly, funny, actually gave a toss about what your students were doing, and always had a cool head on your shoulders no matter what crisis was going on around you. As a student, I valued your (always very sensible) scientific advice, which would invariably inspire me to consider 14 other aspects of my data which I hadn't yet considered. More broadly speaking, I continue to aspire to your sense of responsibility, whether it be cleaning up people's used dishes in the kitchen, or putting your best forward in the teaching arena. In a world where tertiary education is very much a commodity, you have helped me to retain my faith that learning new things is not just something I had grudgingly doled out to me because I purchased it, but something which is fun, inspiring, and may actually (dare I say it) have an impact on the wider world.

While I'm riding the philosophical wave, I'd also like to thank my collaborators. As a researcher who had some of my most interesting findings ”scooped” by another group, I learnt rather early why scientists tend to keep their cards very close to their chest. For that reason, I have great appreciation for those who quickly got on board when approached about investigating a new idea, or were happy to approach me about some of my work. Special thanks goes to Betsy Komives, Ingrid Devries and the rest of the crew at UCSD, Kian Leong Lee, Lorenz Poellinger, Jolene Caifeng Ho and Jia You at the Cancer Science Institute of Singapore, Marie Bogoyevitch at the University of Melbourne, and Grant Booker, Kate Wegener and Iain Murchland at the University of Adelaide. In this category, I also have to mention Dan again, as his response whenever I queried him about sharing information or reagents was always an immediate (and refreshing) "send them whatever they need".

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– you guys got to experience newbie scientist Rachel, and handled it all with aplomb. Karolinee, my comrade in arms, I'm so glad I got to struggle though it all with you! Sam, for his quiet but capable presence, and Sarah Wilkins, who, to this day remains one of the scariest (in a good way, if that's possible) people I've ever met. Words out of your mouth always had unpredictable effects, and never ceased to be entertaining. You were one of the biggest generators of the Peet Lab's social and teaching culture, a feature which I always admired and appreciated. Bec, my moral compass, a genuinely inspiring gal with a tremendous sense of responsibility (all the more amazing because it comes with a healthy dose of cynical humour). TERESA! If there's something that every lab must have, it's a Teresa. Nothing but fun and good humour, I'm greatly looking forward to your return to Oz. Briony, one of those rare creatures who brightens the world around them – I love your curiosity and optimism, 2 things which you share in buckets with everyone else. Our many honours students over the years, including Freya, Michael, Lauren (I will never forget that exchange with Pete ("why are you such a knob?"), and Wai Li (another whose mouth played host to many a surprising sentence). Two of my "own" students, Jackie Lu and Max Tollenaere, both of whom contributed work which ended up in this thesis. As all teachers know, there's nothing better than having a student who's actually engaged in what they're doing, and both put in a great effort while in the lab. Plus, Max, rifling through your lab book to find various experimental details was very funny – I didn't even need Dutch-to-English translation for those rude words! Lastly, to those who currently occupy the Peet Lab as I make my exit: Erin – a rockin' result this year – go girl! Navdeep, someone with a wonderful, cheeky sense of humour who has taught me many interesting things about Indian culture. NatandJay (think, RichandAmy from Zits) the feisty Spanish/Italian duo that always ensure that the lab is filled with noise of some description , not to mention good food. They are lovely individuals – Nat with her humour and incomparable story telling skills (I've seen many an audience in thrall), and Jay, with her own (usually self-aimed) sense of humour, which never fails to make me laugh, even on the crap days. Finally, Ice, another wonderful, upbeat gal who shares many a giggle with me, usually over our inability to pronounce various words in the English or Thai language. Always good fun.

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Author contributions

The following author contribution statement details the contribution of each author to the published article, “Consequences of IkappaB alpha hydroxylation by the factor inhibiting HIF (FIH)”, which can be found in section 6.2.

Statement of Authorship

Title of Paper	Consequences of IkappaB alpha hydroxylation by the factor inhibiting HIF (FIH)	
Publication Status	☑ Published ☐ Submitted for Publication	☐ Accepted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
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Co-1st Author

Name of Co-1st Author (Candidate)	Rachel Hampton-Smith		
Contribution to the Paper	Co-conceived the project, planned experiments, generated data, interpreted experiments, discussed data, edited the paper.		
Overall percentage (%)	20%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the joint primary author of this		
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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Contribution to the Paper
Ingrid Devries	Planned experiments, generated data for Fig 3, interpreted experiments, discussed data.
Melinda Mulvihill	Planned experiments, generated data for Figs 4, 5 and 6, interpreted experiments, discussed data.
Vera Alverdi	Planned experiments, generated data for Fig 2, interpreted experiments.
Daniel Peet	Co-conceived the project, planned experiments, interpreted experiments, discussed data, edited the paper.
Elizabeth Komives	Co-conceived the project, planned experiments, interpreted experiments, discussed data, wrote and edited the paper, acted as corresponding author.

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