Regulation of the \textit{Rgs4} Gene by the Hypoxia Inducible Factors

Sam Olechnowicz

Laboratory of Dr Daniel Peet
School of Molecular and Biomedical Sciences
University of Adelaide
DECLARATION

Name: Sam Olechnowicz

Program: PhD

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 Sam Olechnowicz 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, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission as been granted by the University to restrict access for a period of time.

Signature: ……………………………………………………………

Date: …………………
ABSTRACT .................................................................................................................. 6

ACKNOWLEDGEMENTS ............................................................................................... 7

ABBREVIATIONS ........................................................................................................... 8

1. INTRODUCTION ........................................................................................................... 9

1.1. THE HYPOXIA INDUCIBLE FACTORS ................................................................ 10

1.1.1. Hypoxia and the Hypoxia Inducible Factors .................................................. 10
1.1.2. Post-Translational HIF Regulation ................................................................. 11
1.1.3. Targets of HIF Transactivation ...................................................................... 14
1.1.4. Complex Patterns of HIF Hypoxic Regulation .............................................. 17
1.1.5. Regulation of HIF-α mRNA Transcription by Hypoxia ............................... 20
1.1.6. Post-transcriptional Control of HIF-α Message by Hypoxia ....................... 21
1.1.7. HIF-α Physiological Roles in Mice and Humans .......................................... 24
1.1.8. Effects of the HIF Pathway on Cancer ......................................................... 27
1.1.9. Summary and Preliminary Approach ............................................................ 29

1.2. PRELIMINARY EXPERIMENTS ......................................................................... 30

1.2.1. Novel HIF Target Genes .............................................................................. 30
1.2.2. The PC12/TetON System ............................................................................. 31
1.2.3. Verification of Microarray Data ................................................................... 32

1.3. REGULATOR OF G-PROTEIN SIGNALLING 4 ................................................. 34

1.3.1. G-Protein Coupled Receptors and the RGS Family of Proteins ................. 34
1.3.2. Expression Pattern of RGS4 ....................................................................... 36
1.3.3. Regulation of RGS Genes and Proteins by Hypoxia ................................... 37
1.3.4. Gα-selectivity of RGS4 Function ................................................................. 39
1.3.5. RGS4 Function in the Brain ........................................................................ 40
1.3.6. RGS4 Function in the Cardiovascular and Other Systems ......................... 42

1.4. SUMMARY AND APPROACH ............................................................................. 44

1.4.1. Summary ....................................................................................................... 44
1.4.2. Approach ...................................................................................................... 45

2. METHODS ............................................................................................................... 46

2.1. REAGENTS .......................................................................................................... 47

2.1.1. Commercially Sourced Reagents ................................................................. 47
2.1.2. PCR Primer Sequences ............................................................................... 48
2.1.3. siRNA Duplexes .......................................................................................... 53
2.1.4. Plasmids ....................................................................................................... 54
2.2. EXPERIMENTAL PROCEDURES .............................................................. 54

2.2.1. Tissue Culture .................................................................................... 54
2.2.2. Total RNA Extraction and Northern Transfer ................................. 55
2.2.3. Generation of Radiolabelled Probes ............................................... 56
2.2.4. Hybridisation of Northern Transfer Membrane ............................. 57
2.2.5. Reverse Transcription of mRNA ..................................................... 57
2.2.6. Quantitative PCR ............................................................................. 58
2.2.7. Transfection of siRNA Duplexes .................................................... 59
2.2.8. Western Transfer ............................................................................. 60
2.2.9. Actinomycin D Treatment and mRNA Decay Calculation ........... 61
2.2.10. Mammalian Luciferase Reporter Assays ....................................... 62
2.2.11. Published ChIP-seq Data Alignment and Analysis ....................... 63

3. RGS4 IS RESPONSIVE TO HYPOXIA AND THE HIF PATHWAY .......... 64

3.1. RESULTS ............................................................................................. 65

3.1.1. Preamble .......................................................................................... 65
3.1.2. Northern Blot Analysis of Rgs4 Response to Hypoxia and Mimetics ... 65
3.1.3. Response of Rgs4 in Human Neuroblastoma to Hypoxia and Mimetics 68
3.1.4. Hypoxic Response is Specific for Rgs4 in Neural-like Cells .......... 70
3.1.5. Loss of HIF-1 or HIF-2 Impairs the Response of Rgs4 to 2,2’Dipyridyl 73
3.1.6. HIF-α Knockdown has a Small Effect on Rgs4 Hypoxic Response ... 77

3.2. DISCUSSION ...................................................................................... 79

3.2.1. Hypoxic Response of Rgs4 is Conserved, but Cell Type Specific .... 79
3.2.2. Rgs4 is Regulated by Both HIF Forms ........................................... 82

4. THE MOLECULAR MECHANISM OF RGS4 RESPONSE TO HYPOXIA .................................................. 86

4.1. RESULTS ............................................................................................. 87

4.1.1. Preamble .......................................................................................... 87
4.1.2. Rgs4 Hypoxic Response is Dependent on Transcription ............... 87
4.1.3. The Rgs4 3’UTR does not Confer Hypoxic Regulation ................. 90
4.1.4. Rgs4 is Co-regulated with Other Known HIF Targets .................. 91
4.1.5. Hif1a and Epas1 are Co-regulated with Secondary HIF Targets ..... 93
4.1.6. Reporter Assays do not Detect an Rgs4 Proximal Hypoxia Responsive Enhancer ................................................................. 96
4.1.7. Further Bioinformatic Analyses ...................................................... 103

4.2. DISCUSSION ...................................................................................... 108

4.2.1. The Rgs4 Locus is Likely to be a Direct HIF Binding Target .......... 108
4.2.2. Rgs4 Transcription May be Regulated by a Distant Enhancer ....... 110
The transcriptional response to hypoxia is critically dependent on the Hypoxia Inducible Factors HIF-1 and HIF-2, which have roles not only in development and cellular adaptation to low oxygen levels, but also in diseases such as cancer. Although many HIF target genes have been well-characterised, there is strong evidence that the complete set of HIF responsive genes have not been described. This thesis describes the characterisation of a novel hypoxia responsive gene, *Rgs4*, found by a previous microarray study. *Rgs4* encodes the Regulator of G-protein Signalling 4 (RGS4 protein), which directly inhibits signalling from various G-protein coupled receptors. Hypoxic regulation of *Rgs4* mRNA is observed in neuroblastoma and pheochromocytoma cells derived from rat, mouse and human samples. This response is found to be mimicked by HIF pathway activating chemicals, and occurs in a manner consistent with direct HIF regulation of *Rgs4* transcription. However, hypoxic regulation of this gene is not observed in all cell types that *Rgs4* is expressed in. Reporter gene assays testing 32.9kb of the locus encompassing the *Rgs4* gene failed to detect a hypoxia responsive element, though bioinformatics analysis indicates that *Rgs4* is under the control of distant enhancers outside of the region tested. Further characterisation of the *Rgs4* hypoxic response could help to explain functions of HIF that are currently poorly characterised, such as its effect on catecholamine release and signalling, while the atypical nature of *Rgs4* regulation by HIF may provide a model to discover other as-yet unknown HIF interacting proteins and cell type specific HIF target genes.
Acknowledgements

I would like to acknowledge the guidance and support of my supervisors Dr. Dan Peet and Dr. Murray Whitelaw, who contributed greatly to discussions about the directions and relevance of this research, and set the standard for making the lab a welcoming place to work. I would also like to thank my parents Kathy and Jan, who have nurtured my curious nature and love of science throughout my entire life, and supported me during the difficult stages of completing this thesis. To Sarah, who the one who was always there for me through every aspect of my time as a postgraduate, from science discussions to organising a social culture around the department, and also in dreaming about the future. I would finally like to acknowledge all the others in the MLS building who made it such a conducive place to do research, from the teaching staff such as Lynn, Tony and Garry, to those such as Serge working in the store and TSU, and to those working in the other 3rd floor labs who not only helped with my research by most importantly contributed to the warm and social atmosphere that made coming to the lab every day something to look forward to.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>bHLH</td>
<td>Basic Helix-Loop-Helix</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA (from mRNA)</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>ChIP-seq</td>
<td>ChIP, with detection by deep sequencing</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMOG</td>
<td>Dimethyloxallyl glycine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>DP</td>
<td>2,2’-dipyridyl</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem (cells)</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>HRE</td>
<td>Hypoxia response element</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>mES</td>
<td>Mouse embryonic stem (cells)</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Reverse transcription of mRNA to cDNA, with subsequent qPCR</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcription start site</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
</tbody>
</table>