Regulation of Cardiac Hypertrophy and Metabolism by Regulator of G Protein Signalling 2 (RGS2)

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Abstract

Pathological left ventricular hypertrophy is a maladaptive cardiomyocyte growth response to various cardiovascular conditions such as hypertension, and is a major risk factor for heart failure and stroke. The majority of drugs used to treat cardiovascular diseases target G protein coupled receptors (GPCRs), which are regulated by regulator of G protein signalling (RGS) proteins. RGS2 is a GTPase activating protein which limits \( G_q \) and \( G_s \)-mediated signalling, which are known to play major roles in the development of pathological cardiac hypertrophy. In addition to its G protein effects, we have previously shown that RGS2 can also inhibit protein synthesis and cultured cardiomyocyte growth via a region in its RGS domain, RGS2\(^{eb} \), which inhibits the rate-limiting eIF2B/eIF2 step of protein synthesis initiation. Thus, we hypothesized that the \textit{in vivo} expression of RGS2\(^{eb} \) could limit the development of experimentally induced hypertrophy. Herein, we demonstrate that the \textit{in vivo} cardiomyocyte specific overexpression of RGS2\(^{eb} \) prevents the development of cardiac hypertrophy in a transverse aortic constriction (TAC) model of experimental pressure overload, as well as functional loss and the expression of “fetal” genes associated with hypertrophy and heart failure. Although we further hypothesized that the expression of RGS2\(^{eb} \) in \textit{Rgs2}\(^{−/−} \) mice (which are highly sensitive to hypertrophy and heart failure following pressure overload) could compensate for the loss of endogenous RGS2, we were limited in our characterizations by poor survival rates in \textit{Rgs2}\(^{−/−} \) animals following TAC surgery.

Cardiovascular dysfunction is also known to be directly influenced by obesity and weight gain, which reflects the importance of understanding the mechanisms behind metabolic dysregulation. We have previously demonstrated that \textit{Rgs2}\(^{−/−} \) mice exhibit a lean phenotype on a normal chow diet, but still gain weight on a high fat diet. We thus sought to further characterize the metabolic role of RGS2 \textit{via} Comprehensive Lab Animal Monitoring System (CLAMS) metabolic cages and gene expression analysis of skeletal rather than adipose tissue. Here we report that loss of RGS2 leads to greater use of carbohydrates and significant increases in basal metabolism, as well as increases in associated metabolism-related genes. Altogether, these studies provide new insights into the role of RGS2 in cardiovascular health and metabolism.
Keywords

Regulator of G protein signalling 2 (RGS2), RGS2\textsuperscript{ob}, G protein-coupled receptors (GPCRs), eukaryotic initiation factor 2 (eIF2), eukaryotic initiation factor 2B (eIF2B), cardiac hypertrophy, CLAMS metabolic cages, metabolism, lean phenotype.
Co-Authorship Statement

Chapter 3:

KNL, XL, QPF, and PC designed the experiments. CN generated mouse line, KNL managed all mice colonies. Animal surgeries were conducted by KNL and XL. KNL performed all other experiments and analyzed all data. KNL and PC wrote the manuscript, with revisions and approval from all authors.

Chapter 4:

KNL and PC designed the experiments. KNL, XL, and FLX performed TAC surgeries; KNL and RG conducted osmotic pump implantations. KNL performed all other experiments and analyzed all data. KNL and PC wrote the manuscript.

Chapter 5:

KNL, RG, and PC designed the experiments. KNL and RG conducted CLAMS cage experiments. KNL performed all other experiments and analyzed all data. KNL and PC wrote the manuscript, with revisions and approval from all authors.
Dedication

To my parents and grandparents
Acknowledgments

First, I am incredibly thankful to have had Dr. Peter Chidiac as my doctoral supervisor. The positive research environment that he continuously fosters has given me the opportunity to conduct high quality research, explore my own ideas, and develop as an independent scientist. He has, through his immeasurable patience and sage guidance, instilled within me a core set of technical and intangible skills which I will value for the foreseeable future, and for that I am forever grateful.

I would also like to thank my co-supervisor Dr. Qingping Feng for his guidance and in-depth knowledge of cardiovascular physiology, without which I could not have completed much of my doctoral work. In addition, I am grateful for the generous amount of time that Dr. Sharon Lu dedicated to train me in surgical and lab techniques, as well as for her surgery skills, which were critical for the delicate procedures that were required in our animal models. I am further thankful for the mentorship and experimental help from my advisory committee member Dr. Robert Gros, who was integral to the successful development of many of my projects. I also thank Dr. Thomas Drysdale, my advisory committee chair, who facilitated and contributed invaluable research advice.

I am fortunate to have completed my training within the collegial environment of the Department of Physiology and Pharmacology, and am thankful for the support and advice I have received from various faculty and staff including, among many others, Dr. Frank Beier, Dr. John DiGuglielmo, Murong Liu, Susan McMillan, Dr. Rithwik Ramachandran, Dr. Peter Stathopulos, Chris Webb, and Dr. Lin Zhao.

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provincial government, and the Schulich School of Medicine and Dentistry for the financial support of this thesis.

Finally, I would like to thank my family. My grandparents, aunts, and uncles have always supported me, and I am so very grateful for their love. Ultimately, this thesis could not have been possible without my mother and father. My successes stem directly from their unconditional support, their hard work, and the sacrifices they have made for me. I am beyond fortunate to have them as my parents.
# Table of Contents

Abstract ........................................................................................................................................... i  
Co-Authorship Statement ............................................................................................................. iii  
Dedication ....................................................................................................................................... iv  
Acknowledgments ........................................................................................................................ v  
Table of Contents .......................................................................................................................... vii  
List of Tables .................................................................................................................................. xv  
List of Figures ............................................................................................................................... xvi  
List of Appendices ........................................................................................................................ xvi  
Abbreviations .................................................................................................................................. xix  

1 INTRODUCTION .......................................................................................................................1  
  1.1 Regulator of G protein signalling (RGS) proteins .................................................................2  
  1.2 Regulator of G protein signalling 2 (RGS2) ........................................................................3  
    1.2.1 Expression and regulation of RGS2 ..............................................................................3  
    1.2.2 RGS2\(^{eb}\) regulates mRNA translation ...................................................................4  
  1.3 Signalling pathways of cardiac hypertrophy .......................................................................7  
    1.3.1 Physiological hypertrophy is regulated by PI3K-Akt signalling .................................7  
    1.3.2 Pathological cardiac hypertrophy is regulated by GPCR signalling .........................8  
    1.3.3 Prolonged G\(_{\alpha q/11}\) signalling leads to maladaptive cardiac hypertrophy ........8  
    1.3.4 G\(_{\alpha s}\) signalling mediates β-adrenergic induced hypertrophy ..............................9
1.4 Regulation of protein synthesis in cardiac hypertrophy ........................................... 10
  1.4.1 Overview of protein synthesis ........................................................................ 10
  1.4.2 Regulation of eIF2Bε ..................................................................................... 10
1.5 RGS proteins in the heart ...................................................................................... 11
  1.5.1 Cardioprotective role of RGS2 ........................................................................ 13
1.6 Clinical aspects of pathological left ventricular hypertrophy (LVH) .................... 15
  1.6.1 General characteristics cardiac hypertrophy .................................................. 15
  1.6.2 General overview of LVH in patient populations ........................................... 18
  1.6.3 LVH increases cardiovascular risk ................................................................... 19
  1.6.4 Non-pharmacological interventions ............................................................... 20
  1.6.5 Pharmacological treatments .......................................................................... 21
1.7 Altered RGS2 expression in humans is associated with cardiovascular dysfunction ............................................................................................................. 22
1.8 Animal models of cardiac hypertrophy ............................................................... 23
  1.8.1 Transverse aortic constriction ...................................................................... 23
  1.8.2 Osmotic pump infusion .................................................................................. 24
1.9 Cardiovascular health and obesity ....................................................................... 25
1.10 Regulation of energy balance by GPCRs .............................................................. 25
1.11 Altered G protein signalling in lean and obese phenotypes .................................. 27
1.12 Skeletal tissue is a major site of energy metabolism via thyroid hormone (TH) regulation .................................................................................................................. 27
1.13 TH regulation of metabolism and glucose homeostasis requires deiodinase type 2 (DIO2) for T4-T3 conversion .................................................................29
1.14 Role for RGS2 in metabolic pathways.........................................................30
1.15 Rgs2−/− mice display a lean phenotype and are hyperphagic ......................30
1.16 Diet-induced models of weight gain and loss ..............................................31
  1.16.1 High-fat diets .........................................................................................31
  1.16.2 Dietary restriction ..................................................................................32
1.17 Summary ....................................................................................................32
1.18 References .................................................................................................33

2 HYPOTHESES AND SPECIFIC AIMS .......................................................70

2.1 Specific Aim 1 ..........................................................................................71
  2.1.1 Study 1A: Protective effect of RGS2eb in animal models of cardiac hypertrophy .........................................................................................71
  2.1.2 Study 1B: Rescue effect of RGS2eb in RGS2 knockout animals ..........72

2.2 Specific Aim 2 ..........................................................................................73
  2.2.1 Study 2A: Baseline characterization of the metabolic phenotype ..........74
  2.2.2 Study 2B: Chronic diet restriction model ..............................................74
  2.2.3 Study 2C: Acute diet restriction model ..................................................75

2.3 References ..................................................................................................76

3 CARDIOMYOCYTE SPECIFIC OVEREXPRESSION OF A 37 AMINO ACID DOMAIN OF REGULATOR OF G PROTEIN SIGNALLING 2 INHIBITS
CARDIAC HYPERTROPHY AND IMPROVES FUNCTION IN RESPONSE TO PRESSURE OVERLOAD IN MICE ................................................................. 78

3.1 Introduction .................................................................................................................. 79

3.2 Methods .......................................................................................................................... 81

3.2.1 Generation of myosin heavy chain promoter (MHC) – RGS2\(^{eb}\) transgenic mice ........................................................................... 81

3.2.2 Transverse aortic constriction (TAC) ....................................................................... 82

3.2.3 Assessment of cardiac function ................................................................................. 82

3.2.4 Heart weight/body weight ratios ............................................................................... 82

3.2.5 Histological analysis ................................................................................................. 83

3.2.6 Cardiomyocyte Cell Size and LV wall thickness ....................................................... 83

3.2.7 Immunohistochemistry ............................................................................................. 83

3.2.8 Dot blotting .............................................................................................................. 84

3.2.9 Quantitative reverse transcriptase qPCR ................................................................. 84

3.2.10 Statistical analysis ................................................................................................... 85

3.3 Results ........................................................................................................................... 85

3.3.1 Generation of transgenic mice with cardiomyocyte specific expression of RGS2\(^{eb}\) ........................................................................... 85

3.3.2 Improved cardiac function in RGS2\(^{eb}\) transgenic mice following pressure overload ........................................................................... 86

3.3.3 Cardiac hypertrophy is inhibited in RGS2\(^{eb}\) transgenic mice following pressure overload ........................................................................... 87
3.3.4 Expression of cardiac hypertrophy markers is suppressed in RGS2\textsuperscript{eb} transgenic mice .................................................................88

3.4 Discussion .................................................................................................................97

3.5 Conclusions ................................................................................................................100

3.6 References ................................................................................................................102

4 EFFECTS OF TRANSVERSE AORTIC CONSTRICTION AND DRUG INDUCED EXPERIMENTAL PRESSURE OVERLOAD HYPERTROPHY ON MICE WITH CARDIOMYOCYTE SPECIFIC OVEREXPRESSION OF RGS2\textsuperscript{eb} .......................110

4.1 Introduction ...............................................................................................................111

4.2 Methods ....................................................................................................................113

4.2.1 Generation of \( Rgs2^{-/-} \) mice with cardiac specific overexpression of RGS2\textsuperscript{eb} ..................................................................................113

4.2.2 Transverse aortic constriction (TAC) .................................................................114

4.2.3 Drug treatments .................................................................................................114

4.2.4 Assessment of blood pressure .........................................................................115

4.2.5 Assessment of cardiac function .......................................................................115

4.2.6 Tissue collection ...............................................................................................116

4.2.7 Quantitative reverse transcriptase qPCR .........................................................116

4.2.8 Statistical analysis ............................................................................................116

4.3 Results ....................................................................................................................117

4.3.1 Generation of transgenic mice with cardiomyocyte-specific overexpression of RGS2\textsuperscript{eb} ........................................................................117

xi
4.3.2 Increased mortality in Rgs2−/− mice following pressure overload........117

4.3.3 28 day angiotensin II treatment at 1.44 mg/kg/day..........................118

4.3.4 Decreased cardiac function in Rgs2−/− mice following 28 days of angiotensin II treatment.................................................................119

4.3.5 Expression of fetal genes in LV tissue of 28 day 1.44 mg/kg/day chronic angiotensin II treated mice.................................................................119

4.3.6 14 day angiotensin II treatment at 3.0 mg/kg/day............................131

4.3.7 Effect of twice daily isoproterenol injections.................................131

4.4 Discussion.........................................................................................132

4.5 Conclusions......................................................................................138

4.6 References.......................................................................................139

5 LOSS OF REGULATOR OF G PROTEIN SIGNALLING 2 (RGS2) ALTERS THE EFFECTS OF ACUTE AND CHRONIC DIET RESTRICTION ON METABOLIC OUTCOMES IN C57 BL/6 MICE..................................................145

5.1 Introduction.......................................................................................146

5.2 Methods............................................................................................148

5.2.1 Animals.........................................................................................148

5.2.2 Study design..................................................................................148

5.2.3 Assessment of indirect calorimetry and activity..............................148

5.2.4 Ad libitum studies..........................................................................149

5.2.5 Chronic diet restriction studies......................................................149

5.2.6 Acute diet restriction studies.........................................................150
5.2.7 Microarray analysis.................................................................150
5.2.8 Quantitative reverse transcriptase qPCR .................................150
5.2.9 Statistical analysis..................................................................151
5.3 Results.......................................................................................153
5.3.1 Increased metabolism over time in Rgs2–/– mice on ad libitum diet .......153
5.3.2 Chronic caloric restriction unmasks metabolic differences between Rgs2–/– and WT mice ..........................................................154
5.3.3 Decreased energy conservation in Rgs2–/– mice during and after acute fast ..........................................................155
5.3.4 Altered expression of metabolic genes in Rgs2–/– mice .................166
5.4 Discussion...................................................................................170
5.5 Conclusions.................................................................................173
5.6 References..................................................................................175
6 DISCUSSION AND CONCLUSIONS ..............................................181
6.1 Summary and Discussion..............................................................182
6.1.1 Chapter 3 – Cardiomyocyte specific overexpression of a 37 amino acid domain of RGS2 inhibits cardiac hypertrophy and improves function in response to pressure overload in mice ........................................182
6.1.2 Chapter 4 – Effects of transverse aortic constriction and drug induced experimental pressure overload hypertrophy on Rgs2–/– mice with cardiomyocyte specific overexpression of RGS2eb ........................................183
6.1.3 Chapter 5 – Loss of RGS2 alters the effects of acute and chronic diet restriction on metabolic outcomes in C57 BL.6 mice ........................................184
6.2 Future directions .................................................................185
6.3 Conclusions ........................................................................187
6.4 References .........................................................................189

APPENDICES .............................................................................195

Appendix A: Ethics approval ......................................................196
Appendix B: Copyright approval ................................................198
Appendix C: Supplementary information ....................................199

Curriculum Vitae ......................................................................205
List of Tables

Table 3.1. Hemodynamic parameters in 4 week sham and TAC mice. ................................. 92

Table 4.1. Heart rates for Ang II and saline treated WT, Rgs2+/+(eb), Rgs2−/−, and Rgs2−/−(eb) mice (beats per minute) .................................................................................................................. 123

Table 5.1. Primers for qPCR of metabolism-related genes .................................................. 152

Table 5.2. Ambulatory activity and body weights, ad libitum diet ....................................... 159

Table 5.3. Ambulatory activity and body weights, chronic and fasting diets........................ 160
List of Figures

Figure 1.1. Characteristics of physiological and pathological hypertrophy. ..................... 17

Figure 3.1. Generation of transgenic mice with cardiomyocyte specific expression of RGS2\textsubscript{eb}.................................................................................................................. 89

Figure 3.2. Dot blot detection of histidine-tagged RGS2\textsubscript{eb}................................... 90

Figure 3.3. Pressure overload model of cardiac hypertrophy ............................................. 91

Figure 3.4. Indices of systolic and diastolic function ......................................................... 93

Figure 3.5. Cardiac hypertrophy is reduced in RGS2\textsubscript{eb} transgenic mice following pressure overload................................................................................... 94

Figure 3.6. LV cross sections and cardiomyocytes................................................................ 95

Figure 3.7. Markers of cardiac hypertrophy ....................................................................... 96

Figure 4.1. Breeding schematic for generation of \( Rgs2^{+/+} \text{eb} \) mice .................................. 120

Figure 4.2. Survival and cardiac hypertrophy following 28 days TAC in WT and............. 121

Figure 4.3. Tail-cuff blood pressure measurements for basal, 14 day, and 28 days during saline of Ang II 1.44 mg/kg/day ....................................................................................... 122

Figure 4.4. Heart weight/tibia length ratios and LV mass, 1.44 mg/kg/day Ang II or saline treatment .................................................................................................................. 124

Figure 4.5. LV internal diameters (LVID) at diastole and systole, 1.44 mg/kg/day Ang II or saline treatment ................................................................................................. 125

Figure 4.6. Anterior wall (AW) thickness, diastole and systole, 1.44 mg/kg/day Ang II or saline treatment ................................................................................................................. 126

Figure 4.7. Posterior wall thickness (PW), diastole and systole, 1.44 mg/kg/day Ang II or saline treatment ................................................................................................................. 127
Figure 4.8. LV vol, diastole and systole, 1.44 mg/kg/day Ang II or saline treatment .......... 128

Figure 4.9. Ejection fraction and fractional shortening, 1.44 mg/kg/day Ang II or saline treatment ........................................................................................................................................ 129

Figure 4.10. Markers of cardiac hypertrophy ........................................................................................................................... 130

Figure 4.11. Heart weight/tibia length, LVID, EF, and FS in 3.0 mg/kg/day Ang II or saline treated WT and $R_{gs2^+}$ mice ........................................................................................................................................... 133

Figure 4.12. ISO tests in WT mice, 15 mg/kg s.c. injections ........................................................................................................ 134

Figure 4.13. Lung weight/tibia length and heart weight/tibia length in 14 day saline or 50 mg/kg ISO treated mice ........................................................................................................................................... 135

Figure 5.1. *Ad libitum* diet, indirect calorimetry, VO$_2$ and VCO$_2$ ..................................................................................... 156

Figure 5.2. *Ad libitum* diet, indirect calorimetry, RER and heat .................................................................................................. 157

Figure 5.3. *Ad libitum* diet, food and water intake ..................................................................................................................... 158

Figure 5.4. Chronic diet restriction, indirect calorimetry ............................................................................................................. 161

Figure 5.5. Chronic diet restriction .............................................................................................................................................. 162

Figure 5.6. Acute 16 h fast, indirect calorimetry ........................................................................................................................ 163

Figure 5.7. Acute 16 h fast, indirect calorimetry ........................................................................................................................ 164

Figure 5.8. 16 h fast, food and water intake .............................................................................................................................. 165

Figure 5.9. Gene expression, calcium signalling and metabolic markers ..................................................................................... 167

Figure 5.10. Gene expression, metabolic markers ............................................................................................................. 168

Figure 5.11. GPCR-related gene expression ...................................................................................................................................... 169
List of Appendices

Appendix A: Ethics approval ................................................................. 196

Appendix B: Copyright approval .......................................................... 198

Appendix C: Supplementary figures ...................................................... 199
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>Adc2 and 5</td>
<td>Adenylyl cyclase 2 and 5</td>
</tr>
<tr>
<td>Adb1 and 2</td>
<td>Beta-adrenergic receptor 1 and 2</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANP</td>
<td>Alpha natriuretic peptide</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin receptor blocker</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATP2A1</td>
<td>SERCA1</td>
</tr>
<tr>
<td>ATP5G1</td>
<td>ATP synthase subunit F(0) complex subunit C1</td>
</tr>
<tr>
<td>AW; d and AW; s</td>
<td>Diastolic and systolic anterior wall thickness</td>
</tr>
<tr>
<td>β-AR</td>
<td>Beta adrenergic receptors</td>
</tr>
<tr>
<td>β-MHC</td>
<td>Beta myosin heavy chain</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>BWT</td>
<td>Body weight</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CLAMS</td>
<td>Comprehensive laboratory animal monitoring system</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-diaminobenzidine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DIO1, 2, 3</td>
<td>Iodothyronine deiodinases type I, II, and III</td>
</tr>
<tr>
<td>E&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Effective arterial elastance</td>
</tr>
<tr>
<td>EDP</td>
<td>End diastolic pressure</td>
</tr>
<tr>
<td>EDV</td>
<td>End diastolic volume</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection fraction</td>
</tr>
<tr>
<td>EIF2</td>
<td>Eukaryotic initiation factor 2</td>
</tr>
<tr>
<td>EIF2B</td>
<td>Eukaryotic initiation factor 2B</td>
</tr>
<tr>
<td>EIF2Bε</td>
<td>Eukaryotic initiation factor 2B epsilon subunit</td>
</tr>
<tr>
<td>E&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximal elastance</td>
</tr>
<tr>
<td>ESP</td>
<td>End systolic pressure</td>
</tr>
<tr>
<td>ESV</td>
<td>End systolic volume</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional shortening</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Facilitated glucose transporter 4/solute carrier 2A4 (SLC2A4)</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SLC16A2</td>
<td>Thyroid hormone-specific transporter MCT8</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SV</td>
<td>Stroke volume</td>
</tr>
<tr>
<td>SW</td>
<td>Stroke work</td>
</tr>
<tr>
<td>T3</td>
<td>3,5,3'-triiodothyronine</td>
</tr>
<tr>
<td>T4</td>
<td>3,5,3',5'-tetraiodothyronine (thyroxine)</td>
</tr>
<tr>
<td>TAC</td>
<td>Transverse aortic constriction</td>
</tr>
<tr>
<td>Tau</td>
<td>Relaxation time constant</td>
</tr>
<tr>
<td>TG</td>
<td>Transgenic</td>
</tr>
<tr>
<td>TH</td>
<td>Thyroid hormone</td>
</tr>
<tr>
<td>THRA</td>
<td>Thyroid hormone receptor α</td>
</tr>
<tr>
<td>TL</td>
<td>Tibia length</td>
</tr>
<tr>
<td>UCP3</td>
<td>Mitochondrial uncoupling protein 3</td>
</tr>
<tr>
<td>VCO₂</td>
<td>Volume of carbon dioxide production</td>
</tr>
<tr>
<td>VO₂</td>
<td>Volume of oxygen consumption</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
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1 INTRODUCTION
1.1 Regulator of G protein signalling (RGS) proteins

Agonist binding of G protein coupled receptors (GPCRs) promotes G protein activation, which is achieved by catalyzing GDP–GTP exchange on the Ga subunit; a conformational change in the GTP–bound α subunit is thought to lead to the dissociation of Ga from the stable G-βγ-dimer, thus enabling the regulation of downstream effectors. These signalling events are terminated as a consequence of the intrinsic GTPase activity of the Ga subunit, which hydrolyzes GTP to GDP, resulting in a return to the inactive form of the G protein heterotrimer. The intrinsic GTPase activity of Ga subunits is generally insufficient to explain the observed physiological rates of G protein inactivation (Tang and Insel, 2004), and has been found to be accelerated by GTPase–activating proteins (GAPs) such as regulator of G–protein signalling (RGS) proteins.

RGS proteins are a family of GAPs that bind to G–proteins and inactivate them by increasing the intrinsic rate of GTP hydrolysis. They thus function to limit the intensity and duration of GPCR-initiated signalling, and this is mainly accomplished via a conserved, ~120 amino acid RGS domain that is responsible for their GAP activity (Popov et al., 1997; Ross and Wilkie, 2000). Most RGS proteins act as GAPs towards Ga₁–mediated signalling and can be classified into four subfamilies (R4, RZ, R7, and R12) (Berman, Kozasa and Gilman, 1996; Abramow-Newerly et al., 2006; Jayaraman et al., 2009). There are currently no RGS proteins known to inhibit Gαs activity, although RGS2 has been shown to inhibit Gαs pathways in a GAP-independent manner (Salim et al., 2003; Roy, Baragli, et al., 2006). The GAP activity of RGS proteins is sensitive to a variety of factors such as cations and phospholipids (Wang et al., 1997; Tu, Woodson and Ross, 2001; Cladman and Chidiac, 2002; Ishii, Inanobe and Kurachi, 2002); post-translational modifications can also influence RGS intracellular localization as well as interactions with G proteins and other binding partners (Chidiac and Roy, 2003). Although the RGS domain is necessary and sufficient to confer GAP activity (Popov et al., 1997), other domains can also determine cellular localization, increase substrate specificity, and provide additional activities (Snow et al., 1998; Traver et al., 2004; Wang et al., 2005).
1.2 Regulator of G protein signalling 2 (RGS2)

RGS2 is part of the B/R4 subfamily of RGS proteins (RGS1-5, RGS8, RGS13, RGS16, RGS18, RGS21) which are relatively small and simple in structure (~20-30 kDa), and contain a conserved RGS domain flanked by short N- and C-terminal domains (Riddle et al., 2005). While almost all RGS proteins function as GAPs on G\textsubscript{ai/o} signalling, RGS2 is unique; it demonstrates a low affinity for G\textsubscript{ai/o}, and instead selectively inhibits G\textsubscript{aq} and G\textsubscript{as}–mediated signalling via GAP–dependent and GAP–independent mechanisms (Heximer et al., 1997, 1999; Bernstein et al., 2004; Hague et al., 2005; Roy, Baragli, et al., 2006). The G\textsubscript{aq} selectivity of RGS2 is largely due to three G\textsubscript{aq} amino acid residues (Cys\textsuperscript{106}, Asn\textsuperscript{184}, and Glu\textsuperscript{191}) which appear to reduce RGS2 affinity for G\textsubscript{ai} by steric hindrance, whereas inhibition of G\textsubscript{as} signalling is due at least in part to direct interactions of RGS2 with adenylate cyclases I, II, and VI via its N-terminal domain. (Salim et al., 2003; Roy, Baragli, et al., 2006). In addition, the N-terminal domain of RGS2 can bind other proteins such as spinophilin, muscarinic receptors, \alpha-adrenergic receptors, TRPV6 calcium channels, and tubulin (Bernstein et al., 2004; Hague et al., 2005; Wang et al., 2005; Heo et al., 2006; Schoeber et al., 2006); we have also demonstrated that RGS2 can inhibit protein synthesis in a GAP-independent manner, which will be addressed in detail further below (Nguyen et al., 2009; Chidiac et al., 2014).

1.2.1 Expression and regulation of RGS2

RGS2 is found ubiquitously, with its mRNA expressed in organs such as the heart, brain, lungs, and kidneys, as well as in cell types including pre-adipocytes, vascular smooth muscle cells, osteoblasts, and chondrocytes (Miles et al., 2000; Huang et al., 2003; Larminie et al., 2004). Various signalling pathways and stimuli can induce Rgs2 gene transcription. In human neuroblastoma cells, carbachol stimulation of muscarinic receptors, which are coupled to the phosphoinositide signal transduction system, results in rapid and lasting increases in Rgs2 mRNA levels (Song, De Sarno and Jope, 1999). G\textsubscript{q} agonists such as angiotensin II and phenylephrine, as well as G\textsubscript{s} agonists which increase cAMP levels or intracellular Ca\textsuperscript{2+}, have also been shown to upregulate Rgs2 gene expression in multiple cell types including vascular smooth muscle cells, neonatal cardiomyocytes, and HEK293 cells lines (Grant et al., 2000; Zou et al., 2006). In addition, conserved cAMP response bindings elements in the RGS2
promoter are critical for cAMP-response element binding protein (CREB) binding and RGS2 promoter activation; three single nucleotide polymorphisms (SNPs) have been identified in the human RGS2 promoter CREB binding site in hypertensive patients, with the corresponding point mutations reducing the RGS2 inhibition of forskolin-stimulated adenylyl cyclase activity (Nunn et al., 2010; Xie et al., 2011; Nguyen et al., 2012).

Endogenous RGS2 protein is rapidly degraded via proteasomal mechanisms (Bodenstein, Sunahara and Neubig, 2007; Osei-Owusu et al., 2007). Polymorphisms which enhance RGS2 degradation have been associated with hypertension and cardiovascular dysfunction, and suggest that stabilizing RGS2 protein expression may result in beneficial effects (Bodenstein, Sunahara and Neubig, 2007; Gu, Tirgari and Heximer, 2008; Hahntow et al., 2009; Sjogren et al., 2012). Indeed, more than 30 RGS2 gene polymorphisms have been identified in human hypertensive patients (Yang et al., 2005; Riddle et al., 2006; Semplicini et al., 2006; Zhao et al., 2008); conversely, hypotensive patients with Bartter/Gitelman syndrome have increased RGS2 expression (Calò et al., 2004). In addition, RGS2 deficiency or dysfunction has been linked to anxiety disorders and airway hyper-responsiveness (Xie et al., 2012; Hettema et al., 2013), further highlighting the importance of RGS2 regulation.

1.2.2 RGS2\textsuperscript{eb} regulates mRNA translation

1.2.2.1 Regulation of protein synthesis during translation initiation

The rate of global protein synthesis is controlled primarily at the initiation level, and is divided into multiple stages: (1) GTP-bound eukaryotic initiation factor 2 (eIF2) recognizes and binds initiator tRNA (Met-tRNA\textsuperscript{Met}i), forming the ternary complex eIF2-GTP-Met-tRNA\textsuperscript{Met}i, followed by binding of the complex to the 40S ribosomal subunit; (2) In cap-dependent initiation, the cap structure at the 5’end of mRNA is recognized and bound by eIF4F, which guides the activated 40S subunit to the 5’ end of the mRNA; (3) mRNA-bound 40S travels along the 5’–untranslated end of the mRNA, scanning until it reaches an AUG start codon; (4) Upon start codon detection, the 60S subunit binds to the 40S subunit, initiation factors are released, and the Met-tRNA\textsuperscript{Met}i becomes positioned in the ribosomal P site in preparation for the elongation phase (Jackson, Hellen and Pestova, 2010).
1.2.2.2 Rate limiting step of translation initiation: eIF2 and eIF2B

Of particular importance in the regulation of initiation is the heterotrimeric initiation factor eIF2 which, when bound to GTP, is active and recruits Met–tRNA\textsuperscript{Met\_i} to the ribosomal initiation complex. eIF2 is inactivated by the hydrolysis of GTP; in order for reactivation to occur, GDP must first dissociate. This intrinsically slow process is sped up by the heteropentameric protein eIF2B (\(\alpha – \varepsilon\)), which acts as a guanine nucleotide exchange factor (GEF) by binding to eIF2, and catalyzing GDP/GTP exchange (Abbott and Proud, 2004). Thus, the rate limiting step of initiation is ultimately eIF2B-facilitated GDP/GTP exchange on eIF2 (Rolfe et al., 2005; Singh et al., 2006; Elsby et al., 2011). This makes the modulation of eIF2B activity an important aspect in the regulation of global protein synthesis. Stimuli that promote cell growth may do so by causing the dephosphorylation and consequent activation of eIF2B (Wek, Jiang and Anthony, 2006; Chidiac et al., 2014). Conversely, phosphorylation of eIF2B, as well as eIF2, inhibits their activity and results in the attenuation of cell growth (Hardt et al., 2004).

1.2.2.3 RGS2\textsuperscript{eb} binds to eIF2B and inhibits protein translation

While the G-protein dependent effects of RGS2 are well established, studies have also shown that RGS2 can bind and regulate other targets (Wang et al., 2005; Heo et al., 2006; Schoeber et al., 2006; Zhang and Mende, 2011). Our lab has previously discovered a novel binding partner for RGS2. We have shown that RGS2 can bind to the \(\varepsilon\) subunit of eIF2B, resulting in the inhibition of mRNA translation (Nguyen et al., 2009). By binding to eIF2B\(\varepsilon\), RGS2 inhibits its ability to facilitate GDP dissociation on eIF2, which ultimately leads to the attenuation of \textit{de novo} protein synthesis. This novel, G-protein independent property of RGS2 has been mapped to a 37 amino acid domain (residues 79-115) that is homologous to a region in the \(\beta\) subunit of eIF2 that binds to eIF2B\(\varepsilon\), suggesting that RGS2 may inhibit initiation by competing or otherwise interfering with binding between eIF2 and eIF2B.

We have shown that the eIF2B\(\varepsilon\) binding domain, RGS2\textsuperscript{eb}, to be both necessary and sufficient for the inhibition of protein synthesis by RGS2; a corresponding 37-oligomer peptide based on this region also dose-dependently decreased \textit{in vitro} mRNA translation (Nguyen et al., 2009).
In addition, the cellular expression via adenoviral infection of RGS2\textsuperscript{eb} or full-length RGS2 decreased levels of protein synthesis to a comparable extent (Nguyen \textit{et al.}, 2009). Furthermore, the rate of protein synthesis was increased in cells from RGS2 knockout animals, which suggests that the effect of RGS2 on protein synthesis is physiologically relevant (Nguyen \textit{et al.}, 2009). Thus, RGS2, and more specifically its eIF2B\textepsilon binding domain, appears to be an important regulator of mRNA translation.

This novel mechanism of eIF2B inhibition adds to a group of studies which demonstrate that inhibition of eIF2B leads to decreased protein synthesis. Glycogen synthase kinase 3 (GSK3) can directly phosphorylate eIF2B\textepsilon at serine 540 in intact cells, which inhibits eIF2B GEF activity by up to 80% (Welsh \textit{et al.}, 1998). It has also been shown that the effects of eIF2B on eIF2 can be decreased by the binding of eIF2 to eIF5. The primary function of eIF5 is to promote GTP hydrolysis by eIF2; when present at elevated levels, eIF5 may act to sequester eIF2 from eIF2B, thereby impeding protein synthesis (Singh \textit{et al.}, 2006). Other groups have successfully used eIF2B\textepsilon siRNA (Deng \textit{et al.}, 2008) \textit{in vitro}, as well as adeno-associated viruses (AAVs) \textit{in vivo} to specifically inhibit eIF2B\textepsilon (Guo \textit{et al.}, 2016; Merentie \textit{et al.}, 2016). However, cardiac specific overexpression of full-length RGS2 (which contains the RGS2\textsuperscript{eb} domain) does not appear to confer protective effects against hypertrophy in a pressure overload model (Park-Windhol \textit{et al.}, 2012). As previously mentioned, RGS2 possesses multiple properties not known to be shared by either RGS4 or RGS5, although the latter two are also able to inhibit cardiac hypertrophy when overexpressed (Heximer \textit{et al.}, 1999; Chidiac and Roy, 2003; Roy, Nunn, \textit{et al.}, 2006; Tokudome \textit{et al.}, 2008; Endale \textit{et al.}, 2010; Li \textit{et al.}, 2010; Sethakorn, Yau and Dulin, 2010; Chidiac \textit{et al.}, 2014). Since our studies suggest that the RGS2\textsuperscript{eb} region specifically, and exclusively, inhibits eIF2/eIF2B interactions (and thus protein synthesis) (Nguyen \textit{et al.}, 2009), it is possible that the other properties of RGS2 may have confounded or attributed to the limited cardioprotection that was observed by groups expressing full-length RGS2. Thus in the context of cardiovascular health, the protein synthesis inhibitory effects of RGS2\textsuperscript{eb} could be a major contributor to the reported beneficial effects of RGS2.
1.3 Signalling pathways of cardiac hypertrophy

1.3.1 Physiological hypertrophy is regulated by PI3K-Akt signalling

Physiological and pathological hypertrophies differ not only in terms of phenotype, but can be distinguished by the different signalling cascades that drive them. Exercise or developmentally-induced growth is primarily mediated by growth hormones, and a critical pathway for physiological hypertrophy is the phosphoinositide 3-kinase (PI3K)–Akt pathway (Hunter and Chien, 1999; McMullen and Jennings, 2007). When IGF-1, insulin, and other growth factors bind to their membrane tyrosine receptor kinases, the PI3K isoform p110α is activated, and phosphorylates membrane phosphatidylinositols at the 3’ position of the inositol ring (Dorn and Force, 2005). This phosphorylation results in the recruitment of the protein kinase Akt (also known as PKB) and its activator 3-phosphoinositide-dependent protein kinase-1 (PDK1). Activation of Akt by PDK1 leads to the activation of mammalian target of rapamycin (mTOR), which is a central regulator of protein synthesis via its ability to activate translational machinery and ribosome biogenesis (Dorn and Force, 2005). In addition, Akt can phosphorylate and inhibit the activity of glycogen synthase kinase-3 (GSK-3), a constitutively active negative regulator of cardiac hypertrophy (Haq et al., 2000; Hardt and Sadoshima, 2004; Kerkelä, Woulfe and Force, 2007).

Several studies have demonstrated the importance of PI3K-p110α/PDK1/Akt signalling for adaptive cardiac hypertrophy. Transgenic mice constitutively overexpressing the p110α isoform of PI3K exhibited exercise-induced hypertrophy that did not transition into pathological growth (Shioi et al., 2000). Furthermore, cardiac expression of a mutant dominant-negative p110α prevented exercise-induced growth, but maladaptive hypertrophy still occurred in response to pressure overload (McMullen et al., 2003). Together, these studies demonstrate the critical role that PI3K-p110α/PDK1/Akt signalling plays in the regulation of physiological hypertrophy.
1.3.2 Pathological cardiac hypertrophy is regulated by GPCR signalling

While adaptive hypertrophy is mediated by receptor tyrosine signalling, the critical pathways for maladaptive growth are initiated by G-protein coupled receptors (Dorn and Force, 2005; Heineke and Molkentin, 2006; Hendriks-Balk et al., 2008). GPCRs are seven transmembrane domain proteins involved in signal transmission. With more than 800 members, they represent the largest and most diverse family of cell surface receptors existing in the mammalian genome (Oldham and Hamm, 2008); cardiovascular cells alone possess greater than 100 different GPCRs (Tang and Insel, 2004). GPCRs play a critical role in linking messages from extracellular ligands to signalling pathways within the cell. With a wide variety of activating ligands and with their localization at the cell membrane, GPCRs represent the largest class of drug targets – more than one-third of current drugs on the market act by binding to GPCRs (Ma and Zemmel, 2002; Wise, Gearing and Rees, 2002; Dror et al., 2011).

Isoforms of the $\mathrm{G}_\alpha$ subunit result in four heterotrimeric G protein families: $\mathrm{G}_{\alpha s}$, $\mathrm{G}_{\alpha i/o}$, $\mathrm{G}_{\alpha q/11}$, and $\mathrm{G}_{\alpha 12}$. While both $\mathrm{G}_{q/11}$ and $\mathrm{G}_s$ signalling can result in cardiomyocyte growth, $\mathrm{G}_q$ in particular has been widely shown to play a crucial role in the development of pathological hypertrophy (D’Angelo et al., 1997; Mende et al., 1998; Bernardo et al., 2010).

1.3.3 Prolonged $\mathrm{G}_{q/11}$ signalling leads to maladaptive cardiac hypertrophy

Heterotrimeric $\mathrm{G}_q$ and the functionally similar $\mathrm{G}_{11}$ transduce signals from hypertrophic receptors for angiotensin, endothelin, norepinephrine, and other neurohormones (Rockman, Koch and Lefkowitz, 2002). When activated, the $\mathrm{G}_{q/11}$ subunit recruits phospholipase C beta (PLCβ) to the cell membrane, where it hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2), resulting in the release of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 can then bind to receptors in the sarcoplasmic reticulum (SR), causing calcium release into the cytosol. At this point, two pathways can contribute to a hypertrophic response. Calcium release along with DAG can activate protein kinase C (PKC), which leads to the subsequent inhibition of GSK-3. It can also affect MAP kinase (MAPK) signalling through proteins such
as ERK1/2, JNK and p38 (Frey and Olson, 2003). Alternatively, Ca\(^{2+}\) release can cause the messenger protein calmodulin to activate calcineurin, which dephosphorylates the transcription factor NFAT. Subsequently, NFAT can translocate to the nucleus, and activate the transcription of hypertrophic response genes (Dorn and Force, 2005).

The importance of \(G_{aq/11}\) signalling in the maladaptive hypertrophic response has been demonstrated in several studies. Transgenic mice with cardiac-specific transgenic overexpression of \(G_{aq/11}\) –linked receptors demonstrate severe cardiac hypertrophy and dysfunction, upregulation of the “fetal” gene response, and premature death (D’Angelo et al., 1997; Mende et al., 1998). Conversely, mice overexpressing a dominant-negative \(G_{aq/11}\) peptide or which lacked \(G_{aq/11}\) were resistant to hypertrophy following pressure overload, but still exhibited exercise-induced physiological cardiac growth (Akhter et al., 1997; Wettschureck et al., 2001). Altogether, these studies indicate that \(G_{aq/11}\) signalling is sufficient for pathophysiological hypertrophy to occur.

In addition to PLC\(\beta\)-mediated hypertrophy, \(G_{aq/11}\) signalling can also act through the PI3K-Akt pathway that is normally associated with physiological growth. \(G_{aq/11}\) stimulated PI3K differs from physiological PI3K in that the activated isoform is changed from \(p110\gamma\) to \(p110\alpha\). However, since both types of PI3K can go on to activate Akt and its downstream mTOR and GSK-3 pathways, it is not well understood how these events can result in different forms of hypertrophy (Dorn and Force, 2005).

### 1.3.4 \(G_{as}\) signalling mediates \(\beta\)-adrenergic induced hypertrophy

Although \(G_{aq/11}\) signalling is the major mediator of maladaptive hypertrophy, \(G_{as}\) also plays a significant role in cardiomyocyte growth. In the heart, \(G_{as}\) proteins are mainly coupled to \(\beta_1\)-adrenergic receptors. Upon agonist binding (e.g. isoproterenol), the \(G_a\) subunit activates adenylyl cyclase (AC), which in turn catalyzes the conversion of ATP into the second messenger cAMP (Dorn and Force, 2005). Increased concentrations of cAMP can then activate protein kinase A (PKA) which, among other functions, inhibits GSK-3 and causes an increase in protein synthesis. Overexpression of \(\beta_1\)-receptors in the hearts of transgenic mice has been shown to initially increase contractile function and responsiveness to isoproterenol, which can
initially be seen as beneficial during early heart failure (Bisognano et al., 2000; Engelhardt et al., 2002). However, chronic stimulation of these receptors results in progressive deterioration of cardiac function, as well as increased hypertrophy and fibrosis (Bisognano et al., 2000; Engelhardt et al., 2002; Bernardo et al., 2010). Thus in addition to $G_q$ signalling, $G_s$–mediated cell growth and heart function also contributes to the maladaptive hypertrophic response.

1.4 Regulation of protein synthesis in cardiac hypertrophy

1.4.1 Overview of protein synthesis

In order for either adaptive or maladaptive cardiac hypertrophy to occur, individual cardiomyocytes must increase in size, and such growth requires increased global protein synthesis. Thus, regardless of the initial stimuli and receptors involved, hypertrophic signals will ultimately result in increased mRNA translation/protein synthesis.

Protein synthesis consists of three main stages: initiation, elongation, and termination. During eukaryotic initiation, an initiator methionyl tRNA forms a complex with a 40S ribosomal subunit, and is then positioned at the start codon of an mRNA molecule (Jackson, Hellen and Pestova, 2010). Binding of the 60S ribosomal subunit allows for elongation to occur, during which amino acids are sequentially added to a growing polypeptide chain via specific tRNA molecules. When a stop codon is reached, protein synthesis is terminated by the release of the newly formed peptide chain and separation of the ribosomal-mRNA complex (Proud, 2007).

1.4.2 Regulation of eIF2Bε

As previously mentioned, regeneration of GTP-bound eIF2 requires eIF2B, and specifically the ε subunit, which contains the catalytic domain towards its C-terminus (Welsh and Proud, 1993; Wang et al., 2001; Gomez, Mohammad and Pavitt, 2002). eIF2B can be activated by multiple stimuli that increase mRNA translation, including insulin and growth factors (Welsh and Proud, 1992; Welsh et al., 1996; Kleijn et al., 1998). Overexpression of eIF2Bε has also been shown to enhance growth in neonatal cardiomyocytes (Hardt et al., 2004). The
importance of eIF2B regulation can be seen by the convergence of hypertrophic signals on constitutively active glycogen synthase kinase 3. GSK-3, and more specifically its β isoform (GSK-3β), has been shown to be the predominant kinase for mediating eIF2B phosphorylation (Hardt and Sadoshima, 2002; Hardt et al., 2004). Under resting conditions, GSK-3β phosphorylates the catalytic ε subunit of eIF2B at a highly conserved serine (Ser\(^{540}\)), resulting in the inhibition of eIF2B activity (Hardt et al., 2004). In addition to Ser\(^{540}\), the epsilon subunit of eIF2B has several other regulatory phosphorylation sites (Wang et al., 2001; Proud, 2007), which suggests that its regulation, and consequently that of eIF2, is critical in the control of global protein synthesis.

Hypertrophic signals can promote the phosphorylation and deactivation of GSK-3β. As previously discussed, several pathways in both adaptive (e.g. IGF1-PI3K-Akt) and maladaptive cardiac hypertrophy (e.g. G\(_q\) and G\(_s\)-mediated) can result in the inhibition of GSK-3β. Studies using β-receptor agonists have shown that inhibition of GSK-3β results in greater eIF2B activity and marked increases in cell size and protein synthesis; conversely, overexpression of GSK-3β decreases levels of eIF2B activity (Hardt et al., 2004). In addition, GSK-3β has been found to block hypertrophy in response to the G\(_q\)-agonists endothelin and phenylephrine (Dorn and Force, 2005). Together, these studies not only suggest that GSK-3β is the predominant kinase in eIF2Bε regulation, but demonstrate that regardless of the initial stimuli, eIF2Bε is required for hypertrophy to occur. Thus, targeting eIF2B for inhibition could prevent or decrease the levels of protein synthesis associated with pathological cardiac hypertrophy.

1.5 RGS proteins in the heart

Several RGS proteins are expressed in mammalian hearts and have been detected in both cardiomyocytes and fibroblasts (Kardestuncer et al., 1998; Doupink, Xu and Shinaman, 2001; Riddle et al., 2005; Zhang and Mende, 2011). In failing human hearts, variable expression results have been obtained, although the expression profiles of RGS2, RGS3, and RGS4 are noticeably altered (Riddle et al., 2005). Some groups have found RGS4 mRNA to be increased
but not RGS2 or RGS3 (Mittmann et al., 2002), whereas others have shown an apparent upregulation of RGS3 and RGS4 mRNA (Owen et al., 2001); other groups have identified decreases in RGS2 protein levels (Takeishi et al., 2000). Overall, it appears that RGS2 is normally highly expressed in the ventricles, whereas RGS4, RGS5, and RGS10 are mainly localized to the atria (Doupnik, Xu and Shinaman, 2001; Mittmann et al., 2002; Larminie et al., 2004; Gu et al., 2009; Miao et al., 2016), suggesting that targeting RGS2 is physiologically relevant.

Animal overexpression and knockout models have demonstrated the cardiovascular roles of various RGS proteins. Gene expression studies have found RGS2, RGS3, and RGS5 to be the most highly expressed RGS proteins in the myocardium, (Larminie et al., 2004), and RGS4 and RGS6 to be concentrated in the sinoatrial node (Grillet et al., 2005; Cifelli et al., 2008, Yang et al., 2010). A cardiac mouse model with transgenic overexpression of RGS4 transiently decreased cardiac hypertrophy and heart failure in Gαq− overexpressing mouse hearts (Rogers et al., 2001), although it should be noted that endogenous levels of RGS4 are virtually absent in the ventricular myocardium (Grillet et al., 2005; Cifelli et al., 2008). An overexpression model of RGS5 was protected against pressure overload-induced experimental cardiac hypertrophy and fibrosis, whereas marked cardiac hypertrophy, fibrosis, and dysfunction occurred in RGS5 knockout mice (Li et al., 2010). Recently, RGS10 was found to be downregulated in failing mouse hearts; similarly to RGS5, cardiac-specific overexpression led to decreased cardiac hypertrophy and fibrosis following experimentally induce pressure overload (Miao et al., 2016).

RGS proteins also play an essential role in regulating heart rate. Knock-in mice expressing a RGS–resistant form of Gai2 displayed enhanced carbachol–induced bradycardia (Fu et al., 2006, 2007). RGS4 and RGS6 knockout models also suggest key roles in regulating parasympathetic heart rate control, as their loss resulted in severe bradycardia in response to in vivo parasympathetic stimulation (Cifelli et al., 2008; Posokhova et al., 2010; Yang et al., 2010). RGS2 in particular has been shown to play a significant role in cardiovascular function.
1.5.1 Cardioprotective role of RGS2

Because of the critical roles that G\textsubscript{αq} and G\textsubscript{αs} signalling play in the induction of pathological hypertrophy, the inhibitory effects of RGS2 on these signals makes it a potential target in the study of cardiovascular disease (Heineke and Molkentin, 2006; Hendriks-Balk et al., 2008). In addition to the heart, RGS2 is expressed in the brain, vasculature and kidneys (Kehrl and Sinnarajah, 2002). Along with RGS1, 4, and 5, RGS2 is detectable at both the mRNA or protein level in endothelial or vascular smooth muscles cells of the arterial system (Bondjers et al., 2003; Heximer et al., 2003; Wang et al., 2008; Timofeeva et al., 2009). Rgs2\textsuperscript{-/-} mice have been reported to exhibit elevated blood pressures (Heximer et al., 2003), and G\textsubscript{q} -mediated smooth muscle contraction is enhanced in several regions of the vasculature (e.g. mesenteric and renal arteries) (Hercule et al., 2007; Osei-Owusu et al., 2007; Osei-Owusu and Blumer, 2015). Interestingly, although global deletion of RGS2 has been observed to induce hypertension, vascular smooth muscle or endothelium-specific deletion of RGS2 does not appear to increase blood pressure (Osei-Owusu et al., 2012). However, transplanting Rgs2\textsuperscript{-/-} kidneys into wild type recipient mice is sufficient to induce hypertension, with the reverse transplantation (i.e. WT kidneys transplanted in Rgs2\textsuperscript{-/-} mice) “rescuing” Rgs2\textsuperscript{-/-} animals from an observed hypertensive phenotype (Gurley et al., 2010), suggesting that renal RGS2 is important in maintaining normal levels of blood pressure.

RGS2 appears to be necessary for the antihypertrophic and vasodilatory effects of cGMP and nitric oxide (Sun et al., 2005; Takimoto et al., 2009). Studies in cultured cardiomyocytes have shown that hypertrophy caused by prolonged G\textsubscript{αq}-coupled receptor stimulation, such as that induced by phenylephrine (PE), can be blocked by the overexpression of RGS2 (Hao et al., 2006; Zou et al., 2006). Moreover, RGS2 negatively regulates angiotensin II-induced signalling in cardiac fibroblasts (Zhang et al., 2011). RNAi–mediated knockdown of RGS2 also leads to increased endothelin and phenylephrine–induced cardiomyocyte hypertrophy (Zhang et al., 2006, Nunn et al., 2010, Zou et al., 2006). A similar effect has been seen against G\textsubscript{αs} mediated cardiomyocyte hypertrophy induced by isoproterenol (ISO) (Nunn et al., 2010).

The cardiovascular role of RGS2 has been further demonstrated in knockout animal studies. Under normal conditions, the absence of RGS2 appears to have minimal effects on cardiac
function, although studies have shown $Rgs2^{-/-}$ or $Rgs2^{+/-}$ mice to be hypertensive (Heximer et al., 2003; Tang et al., 2003). However, experimentally induced pressure overload causes marked hypertrophy, heart failure, and death, as well as increased expression of cardiac fetal genes in RGS2 knockout mice (Takimoto et al., 2009). In addition, $Rgs2^{-/-}$ mice have been shown to have increased susceptibility to atrial fibrillation (AF) via enhanced M3 muscarinic receptor activity, which is known to be involved in vagally induced atrial fibrillation (Tuomi, Chidiac and Jones, 2010; Jones, Tuomi and Chidiac, 2012).

Endogenous RGS2 protein is rapidly degraded via proteasomal mechanisms (Bodenstein, Sunahara and Neubig, 2007), and low RGS2 protein levels have been associated with cardiovascular dysfunction and hypertension in human patients (Heximer et al., 2003; Tsang et al., 2010); RGS2 is also selectively downregulated in pressure overload mouse models of cardiac hypertrophy and heart failure (Zhang et al., 2006), notwithstanding its acute upregulation in cardiomyocytes in response to hypertrophy-inducing Gq and Gs-coupled receptor stimulating agonists (Zou et al., 2006; Nunn et al., 2010). These observations suggest that overexpressing, or stabilizing RGS2 protein expression may result in cardioprotective effects. Digoxin and other cardiotonic steroids, which are used to treat atrial fibrillation and heart failure (Gheorghiade, Adams and Colucci, 2004), have been shown to act as selective stabilizers of RGS2 in vitro (Sjogren et al., 2012). Digoxin has also been shown to be cardioprotective in a drug–induced model of cardiac hypertrophy and injury, an effect which was lost in similarly treated RGS2 knockout mice (Sjögren et al., 2016).

Surprisingly, cardiac specific overexpression of full-length RGS2 does not appear to offer substantial cardioprotection against either hypertrophy or dysfunction in a pressure overload mouse model (Park-Windhol et al., 2012). RGS2 possesses multiple properties not known to be shared by either RGS4 or RGS5 (Chidiac et al., 2014), including low affinity for $G_{ai/o}$ (Heximer et al., 1999), ability to inhibit $G_s$-stimulated adenylyl cyclase signalling (Roy, Baragli, et al., 2006), nuclear localization (Chidiac and Roy, 2003), and the apparent ability to promote apoptosis (Endale et al., 2010). In addition, although RGS2 is selectively upregulated in cultured cardiomyocytes following $G_{aq}$ or $G_{as}$ stimulation (Hao et al., 2006; Zou et al., 2006; Nunn et al., 2010), it is progressively downregulated in in vivo models of hypertrophy and
heart failure (Zhang et al., 2006). Since complete ablation of RGS2 signalling is unambiguously detrimental (Takimoto et al., 2009), it follows that optimal RGS2 levels under pathological conditions may be lower than those needed under basal conditions.

Our lab has also shown that expression of either full-length RGS2 or RGS2\(^{eb}\) can both block increases in cardiomyocyte size induced by phenylephrine (PE) or isoproterenol (ISO) (Zou et al., 2006; Chidiac et al., 2014). In addition, the reactivation of “fetal” genes such as ANP and \(\beta\)-MHC, which are indicators of pathological hypertrophic growth, were suppressed in cardiomyocytes expressing RGS2\(^{eb}\) (Chidiac et al., 2014). An important distinction between full length RGS2 and RGS2\(^{eb}\) is that while the latter prevents hypertrophy, it does not have GAP activity for G\(_{\alpha q}\), and also seems to have no inhibitory effect on ISO-induced G\(_{\alpha s}\) signalling (Nguyen et al., 2009; Chidiac et al., 2014). This indicates that in addition to the established role of the RGS2 GAP domain as an inhibitor of G-protein and GPCR signalling, the RGS2\(^{eb}\) region further regulates G-protein induced hypertrophy through its binding to eIF2B\(\varepsilon\), which leads to the direct, G-protein independent inhibition of protein synthesis.

1.6 Clinical aspects of pathological left ventricular hypertrophy (LVH)

1.6.1 General characteristics cardiac hypertrophy

During fetal development, mammalian cardiomyocytes rapidly proliferate; this growth ceases during the perinatal period, and the heart is thus generally regarded as a post-mitotic organ, with further increases in heart mass thought to be mainly achieved through increases in cell size, i.e. hypertrophy (Ahuja, Sdek and MacLellan, 2007; Shimizu and Minamino, 2016). In the adult heart, various intrinsic and extrinsic stimuli can increase biomechanical stress and lead to a thickening of ventricular heart walls. Depending on the stimuli, either physiological or pathological hypertrophy can occur (Fig. 1.1). Physiological cardiac hypertrophy mainly develops in response to growth of the body (e.g. childhood growth) or chronic exercise.
training, whereas pathological cardiac hypertrophy occurs in a disease setting (Levy et al., 1990).

Physiological and pathological hypertrophy can be further subclassified as either concentric or eccentric hypertrophy (McMullen and Jennings, 2007). During concentric hypertrophy, which is induced by pressure overload on the heart, sarcomeres in cardiomyocytes are added in parallel, leading to an increase in myocyte cell width and a subsequent increase in heart wall thickness; this results in hearts with thick walls and relatively small ventricular cavities (Grossman, Jones and McLaurin, 1975; Bernardo et al., 2010). In a physiological context, concentric hypertrophy develops following isometric or static exercise such as weight lifting, which involves developing muscular tension against resistance with little movement (Pluim et al., 2000; McMullen and Jennings, 2007). Pathological stimuli such as hypertension or aortic stenosis similarly cause pressure overload on the heart and the development of concentric hypertrophy (Frey and Olson, 2003). In contrast, eccentric hypertrophy results in sarcomeres being added in series to increase myocyte cell length, leading to relatively thinner walls and larger ventricular cavities (Bernardo et al., 2010). Isotonic exercise, such as running and swimming, involves the movement of large muscle groups; vasodilation of the skeletal vasculature produces eccentric hypertrophy due to increased venous return to the heart, which results in volume overload (Zak, 1984; McMullen and Jennings, 2007). Pathological stimuli such as aortic regurgitation and arteriovenous fistulas can increase diastolic wall stress, and also result in eccentric hypertrophy (Grossman, Jones and McLaurin, 1975; Pluim et al., 2000).

While both physiological and pathological hypertrophies are characterized by an increase in cardiomyocyte size due to increased global protein synthesis, the two conditions differ in multiple aspects. In physiological hypertrophy, cardiomyocytes increase in size but display a normal phenotype, and this is associated with normal cardiac function (Shimizu and Minamino, 2016). Fatty acid oxidation is the main metabolic pathway for ATP production, with the heart capable of switching to glucose and lactate metabolism depending on workload (van der Vusse et al., 1992). In contrast, pathological hypertrophy results in a switch to increased glucose metabolism and structural changes such as increased fibrosis, as well as functional abnormalities (Conrad et al., 1995; Bernardo et al., 2010). Pathological hypertrophy
Figure 1.1. Characteristics of physiological and pathological hypertrophy.

- Normal cardiomyocyte
  - Volume overload
  - Pressure overload
  - Eccentric growth
    - Physiological hypertrophy
      - Endurance training
    - Pathological hypertrophy
      - Valvular disease
  - Concentric growth
    - Physiological hypertrophy
      - Strength training
    - Pathological hypertrophy
      - Hypertension
      - Aortic constriction

Modified from Benardo et al. 2010, Pharmacol Ther
The Noun Project
is also characterized by a return to the “fetal” gene program, which includes increased expression of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and α-skeletal actin, as well as alterations in the cardiac contractile proteins α- and β-myosin heavy chain (Hannan et al., 2003; Bernardo et al., 2010; Taegtmeyer, Sen and Vela, 2010; Kuwahara, Nishikimi and Nakao, 2012). Maladaptive hypertrophy can drastically affect cardiovascular health. Aside from age, left ventricular hypertrophy is the most potent predictor of poor cardiovascular outcomes in hypertensive patients, and is an independent risk factor for sudden death, stroke, coronary heart disease, and heart failure (Levy et al., 1990; Cipriano et al., 2001; Gardin et al., 2001; Verdecchia, Carini, et al., 2001; Verdecchia, Porcellati, et al., 2001; Gradman and Alfayoumi, 2006). With cardiovascular disease as the leading cause of mortality in many nations (Schou et al., 2013), identifying regulators of pathological hypertrophy is of great interest and importance.

1.6.2 General overview of LVH in patient populations

Since the left ventricle is responsible for pumping oxygenated blood throughout the body, the majority of ventricular hypertrophy in a disease setting develops in the left myocardium. Most LVH develops in early response to chronic hypertension; patients with transient elevations in blood pressure, such as during mental stress or exercise, are also prone to hypertrophy (Daniels, Meyer and Loggie, 1990; Schnall et al., 1990). Although hypertension is the leading cause of LVH, factors other than blood pressure can also be important in the development of hypertrophy, including angiotensin II and catecholamine levels, body mass or obesity, genetic background, age of onset of high blood pressure, and insulin sensitivity (Simpson, 1983; Levy et al., 1988; Sasson et al., 1993; Schunkert et al., 1994; Ichikawa et al., 1996; Re, 2003). In addition, genetics may play a role in the development of LVH; mutations in genes encoding sarcomeric proteins have been shown to directly correlate with hypertrophic cardiomyopathy (Maron, 2002), and some patients with mild hypertension appear genetically predisposed to LVH development (Liebson et al., 1993; Martinez et al., 2003).

The test of choice for accurate estimation of patient LV heart mass comes from 2D echocardiography (Lorell and Carabello, 2000); although cardiac MRI is the gold standard test for LVH due to its accuracy and reproducibility, it is cost prohibitive for regular clinical use
(Bottini et al., 1995; Bauml and Underwood, 2010). In hypertensive patients, LVH usually presents as an increase in wall thickness, which may or may not be accompanied by an increase in the size of the LV cavity (Douglas and Kaplan, 2017). Interestingly, patients without an increase in absolute LV mass but with an increase in their wall thickness–to–cavity diameter ratio (concentric remodelling) have the same adverse risk for cardiovascular disease and death as patients who develop concentric hypertrophy (increase in both mass and wall thickness–to–cavity diameter ratio) (van Hoeven and Factor, 1990; Jain et al., 1996). While hypertension induces pressure overload on the heart, concurrent volume overload due to coronary artery disease or valvular conditions can further increase LVH and reduce cardiac function (Zabalgoitia et al., 2001).

1.6.3 LVH increases cardiovascular risk

Diagnosed left ventricular hypertrophy comes with an increased risk of multiple cardiac events including angina and myocardial infarction (MI), arrhythmias, stroke, heart failure, and death (Levy et al. 1990; Koren et al. 1991; Verdecchia, Porcellati, et al. 2001; Haider et al. 1998; Drazner et al. 2004; D Levy et al. 1989). Increases in cardiovascular risk have been found to be directly related to the degree of increase in LV mass; this effect is also independent from that of blood pressure (Levy et al. 1990; Koren et al. 1991). In a study of ~1000 patients over 50 years of age with primary hypertension and no previous cardiovascular events, LVH was associated with a 40% increased risk for a major cardiovascular event (e.g. MI, severe heart failure, severe kidney failure) even after adjustment for other risk factors (Verdecchia, Carini, et al., 2001). In the Framingham Heart Study, which originally followed more than 5000 men and women between the ages of 30 and 62 who had not yet developed overt symptoms of cardiovascular disease, a subset population of ~3000 individuals over the age of 44 were found to have a 22% prevalence of LVH; even after adjusting for other risk factors, these patients were found to be twice as likely to suffer from sudden death (Haider et al., 1998).

LVH can cause electrophysiological alterations such as altered repolarization of the heart, non-uniform action potential prolongation, and early afterpotentials; this can lead to an increased risk of atrial fibrillation, ventricular tachycardia, arrhythmias, and even sudden death (Gardin et al., 2001; Okin et al., 2006; Sarganas et al., 2014). Increases in cardiac risk associated with
LVH may also arise in part due to myocardial ischemia, which can be induced by multiple factors (Douglas and Kaplan, 2017). Enlarged cardiomyocytes, and thus increased muscle mass, can limit the ability of coronary arteries to properly dilate in response to vasodilatory stress (e.g. during exercise) or decreased perfusion (e.g. during an arrhythmic event); endocardial capillaries may also be directly compressed (Beache et al., 2001). These factors can result in adverse clinical implications as well as decreases in coronary flow reserve (the ratio between resting and maximal coronary artery blood flow), an important indicator of cardiovascular risk (Douglas and Kaplan, 2017). In patients with LVH, coronary occlusion events are associated with greater degrees of myocardial infarction and increased mortality rates (Carluccio et al., 2000). In cases of sudden cardiac death, patients with LVH and hypertension tend to have less extensive coronary artery disease than normotensive cases of sudden death, suggesting that hypertrophied myocardium may be more susceptible to the effects of ischemia (Burke et al., 1996). Progressive LVH results in deleterious ventricular remodelling as well as depressed systolic and diastolic ventricular functions (Norton et al., 2002). If improperly treated, LVH may ultimately lead to the development of irreversible heart failure, and is one of the greatest independent risk factors for this condition (Cipriano et al., 2001).

1.6.4 Non-pharmacological interventions

Strategies to manage hypertension and co-morbidities such as obesity can contribute to lowering LV mass by reducing pressure overload on the heart. A high prevalence of hypertension in obese patients (>60%) accounts for more than 60% of incident hypertension (a systolic/diastolic blood pressure greater than 140/90 mmHg) in women and almost 80% of incident hypertension in men. Regression of LVH in patients with primary hypertension has been shown to occur with weight loss and exercise. Sympathetic signalling mechanisms play a well-established role in the development of hypertrophy, and weight loss in obese patients has been reported to reduce indices of sympathetic activity (e.g. plasma and urinary norepinephrine excretion, resting heart rate) (Ostman-Smith, 1981; Reisin et al., 1983; Sen, 1983). Weight reduction also decreases plasma aldosterone levels and renin activity, components of the renin-angiotensin II system which have been implicated in the development
of hypertension and LVH (Iwai, Shimoike and Kinoshita, 1995; Lambert et al., 2014; Seravalle et al., 2014). In patients with hypertension and measurable LVH, calorie restriction-induced weight loss alone can decrease left ventricular mass by up to 20% (MacMahon, Wilcken and Macdonald, 1986). Thus, managing patient weight through diet and exercise can help limit the development of LVH. In addition to weight loss, sodium restriction can inhibit LVH progression. Obese patients often have hyperinsulinemia (excess levels of circulating insulin) leading to increased sodium reabsorption through the renal tubules, which can exacerbate already elevated blood pressures (Jula and Karanko, 1994; Kim et al., 2015). Recommended daily amounts of sodium range between 1000 – 1500 mg; average Canadians consume around ~3400mg of sodium daily, with many eating well in excess of 5000 mg per day (Government of Canada, 2013). Dietary sodium restriction (<1600 mg/day) has been shown to reduce LVH through decreases to blood pressure and body fluid retention in patients with moderate primary hypertension and above average LV mass (Jula and Karanko, 1994). However, although diet and weight loss may markedly benefit patients, clinical studies have demonstrated that such lifestyle modifications are difficult to maintain in the long term (Whelton et al., 1992; Stamler, 1997; Hedayati, Elsayed and Reilly, 2011). Thus, pharmacological intervention is often required to moderate the progression of LVH and associated hypertension.

1.6.5 Pharmacological treatments

Regression of LVH has been shown with multiple drugs including angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), some calcium channel blockers (e.g. verapamil, amlodipine, and diltiazem), renin inhibitors (e.g. aliskiren), and some sympatholytic agents (e.g. alpha–blockers and methyldopa) (Franz, Tönnesmann and Müller, 1998; Okin et al., 2006; Cuspidi, Negri and Zanchetti, 2008; Os et al., 2008; Solomon et al., 2009). Direct vasodilators appear to be largely ineffective in regards to LVH regression, which may be due to reflex stimulation of angiotensin II and norepinephrine release (both of which can directly promote the development of LVH) (Klingbeil et al., 2003). Although ARBs, calcium channel blockers, and ACE inhibitors produce significantly more LVH regression than diuretics and beta blockers, there is currently no evidence that a more rapid regression of LVH is associated with improved long-term health outcomes (Kaplan and Douglas, 2017).
ARBs have been demonstrated to be the most efficacious class of drugs for reducing LV mass (11% reduction in LV mass), followed by calcium channel blockers (11%), ACE inhibitors (10%), diuretics (8%), and beta-blockers (6%) (Klingbeil et al., 2003; Bauml and Underwood, 2010). An important component in LVH development is myocardial fibrosis, which compromises cardiac function via initial diastolic dysfunction followed by progressive systolic dysfunction (Weber et al., 1990); angiotensin II has been shown to induce the development of fibrosis in the myocardium of hypertensive patients (González et al., 2002), and may be an additional mechanism of action by which ACE inhibitors and ARBs exhibit their beneficial effects in cardiac hypertrophy. As such, ACE inhibitors and ARBs are recommended as first-line treatments for LVH, unless contraindicated in the individual patient (Bauml and Underwood, 2010).

1.7 Altered RGS2 expression in humans is associated with cardiovascular dysfunction

Various studies have suggested a potential role of RGS2 dysfunction in human hypertension. Human skin fibroblasts and peripheral blood mononuclear cells from hypertensive patients display decreased RGS2 mRNA expression, increased Ang II-induced MAPK/ERK phosphorylation, and elevated Ca\(^{2+}\) release from internal stores (Semplicini et al., 2006). Multiple Rgs2 SNPs have been identified in genetic groups from China (Zhao et al., 2008; Zhang et al., 2010), Japan (Yang et al., 2005), the Netherlands (Riddle et al., 2006), and the United States (Hettema et al., 2013). Destabilizing N-terminal mutations appear to increase RGS2 susceptibility to arginylation/ubiquitination and subsequent proteasomal degradation (Bodenstein, Sunahara and Neubig, 2007), while other SNPs can affect RGS2 localization and impair GAP activity (Gu, Tirgari and Heximer, 2008). In contrast, RGS2 overexpression has been found in patients with Bartter/Gitelman syndrome, a kidney disorder caused by loss of function mutations of the thiazide-sensitive sodium-chloride symport that is characterized in part by hypotension and an increased risk for ventricular arrhythmia (Deschenes, Feldmann and Doucet, 2002; Foglia et al., 2004; Knoers and Levchenko, 2008). The dysfunction of RGS2 in patients suggests that its study in relation to hypertension and hypertrophy may be...
clinically relevant, and that stabilizing its expression or function may increase or decrease blood pressure; indeed, stabilizing RGS2 expression appears to be beneficial in *in vitro* and in animal models of cardiac hypertrophy (Sjögren, Blazer and Neubig, 2010; Sjogren *et al.*, 2012; Sjögren *et al.*, 2016), and suggests that RGS2 could be a potential target for drug discovery in cardiovascular disease.

1.8 Animal models of cardiac hypertrophy

1.8.1 Transverse aortic constriction

Transverse aortic constriction (TAC) in murine animals is a well-established technique of experimental pressure overload on the heart to induce cardiac hypertrophy and dysfunction, and has been used in multiple studies to examine the role of RGS2 in cardiovascular health (Wang *et al.*, 2008; Takimoto *et al.*, 2009; Takimoto, 2011; Park-Windhol *et al.*, 2012) Briefly, following anaesthesia and intubation, the chest cavity is entered via the second intercostal space through a small incision, and aortic constriction is performed by tying sutures against a needle to yield a narrowing of the aorta once the needle is removed (Rockman *et al.*, 1991). Since its initial development (Rockman *et al.*, 1991), TAC has been extensively used as a model of human cardiovascular disease and for the study of signalling processes involved in hypertrophic responses and heart failure. In comparison to other experimental models of heart failure, such as coronary artery ligation (which results in complete occlusion of the vessel), TAC provides a more gradual model of cardiac hypertrophy development (deAlmeida, van Oort and Wehrens, 2010), which can be modified by both the duration (Mohammed *et al.*, 2012) and severity of the constriction (*i.e.* the gauge of the needle); TAC initially induces compensatory cardiac hypertrophy, which can often present as temporarily enhanced cardiac contractile function. Over time, chronic pressure overload leads to maladaptive cardiac hypertrophy and fibrosis, which can ultimately devolve into heart failure and cardiac dilation (Patten and Hall-Porter, 2009).

A major advantage of TAC is the ability to measure the pressure gradient that is created across the aortic stricture (Patten and Hall-Porter, 2009); since the constriction is performed on the
aorta between the left and right carotid arteries, arterial pressures (as measured by conductance catheters) should be measurably higher in the right carotid artery, thus validating the success of TAC surgery. The utility of this model can be seen from the promising and novel therapeutics targets that have been identified through its use (Braz et al., 2004; Wilkins et al., 2004; Takimoto et al., 2005; Jeong et al., 2006)

1.8.2 Osmotic pump infusion

Although the TAC method is a highly reproducible (Rockman et al., 1991) and useful model of cardiac hypertrophy and heart failure, the acute onset of severe hypertension via aortic stricture may lack direct clinical relevance (Patten and Hall-Porter, 2009). Multiple studies have used continuous drug infusion to induce systemic hypertension (and thus hypertrophy) via angiotensin II (Bueno et al., 2002; Freund et al., 2005; Kee et al., 2006), which is arguably a more clinically relevant model in light of the major role of the renin-angiotensin system (RAS) in the development of human hypertension and cardiac hypertrophy (Weber and Brilla, 1991; Dostal and Baker, 1999). Other groups have chronically infused isoproterenol to stimulate β-adrenergic signalling, and often do so in conjunction with angiotensin and/or TAC models (Soonpaa and Field, 1994; Kudej et al., 1997; Saadane, Alpert and Chalifour, 1999; De Windt et al., 2001; Bueno et al., 2002; Freund et al., 2005).

Following the initial development of an osmotic pump concept (Rose and Nelson, 1995), simplified mini-osmotic pumps have been designed which can systemically deliver sustained doses of desired drugs in a relatively non-invasive manner. Briefly, osmotic pumps operate via an osmotic pressure difference between a salt sleeve that surrounds a flexible and impermeable reservoir (containing a desired drug/solution) and the environment in which the pump is implanted (e.g. subcutaneous or intraperitoneal) (Alzet, 2017). The high osmolality of the salt sleeve draws water through a semipermeable membrane into the pump, leading to compression of the drug-containing reservoir; the drug/solution is thus displaced out of the pump (through a flow moderator) at a controlled rate which is predetermined by the water permeability of the pump’s outer membrane (Theeuwes and Yum, 1976).
1.9 Cardiovascular health and obesity

Cardiovascular dysfunction is known to be directly influenced by obesity and weight gain, which are major risk factors for cardiovascular diseases such as coronary heart disease, atrial fibrillation, hypertension, venous thromboembolism, and congestive heart failure (Klein et al., 2004; Poirier et al., 2006; Tedrow et al., 2010; Wattanakit et al., 2012). In 2011 to 2012, only 65% of children and 31% of adults in the United States met body mass indexes (kg/m²) which correlated with ideal cardiovascular health (Lloyd-Jones et al., 2010). Conversely, 32% of US children and 69% of adults are currently estimated to be overweight or obese (Mozaffarian et al., 2016). The health and economic costs of obesity and its related co-morbidities, such as cardiovascular diseases, are substantial. In 2008, the estimated medical cost of obesity was $147 billion; costs related to the current prevalence of obesity and weight gain in children and adolescents is projected to be $254 billion ($46 billion in direct medical costs, and $208 billion in lost productivity due to morbidities and mortality) (Finkelstein et al., 2009; Lightwood et al., 2009). Although addressing obesity requires a multifactorial response involving clinical, biomedical, and socio-economic approaches, we will focus on examining the physiological mechanisms underlying weight regulation and metabolism, which may provide beneficial insights into treatments and novel drug targets.

1.10 Regulation of energy balance by GPCRs

Multiple GPCRs have been shown to be involved in energy metabolism. Adiposity is determined by energy intake (e.g. food intake) versus energy expenditure (e.g. physical activity, adaptive thermogenesis, basal metabolism), and possible sources of energy imbalance in cases of obesity or conversely, loss of body weight, could come from dysfunctions in food intake/nutrient absorption, lipid storage, metabolic rate, physical activity, or CNS-related changes.

Several studies have demonstrated the involvement of a vast range of GPCR signalling in appetite and satiation. Brain cannabinoid receptors play a physiological role in the control of
appetite and body weight, and cannabinoid CB₁ receptor antagonists reduce appetite and decrease body weights in non-obese Wistar rats (Colombo et al., 1998). Patients with eating disorders exhibit several clinical features and biologic findings indicative of serotonin (5-hydroxytryptamine/5-HT) dysregulation (Brewerton, 1995), and ghrelin infusion has been shown to enhance appetite and increase food intake in humans (Wren et al., 2001). Furthermore, histamine, which acts as a central neurotransmitter, acts as an appetite suppressant via stimulation of histamine H₁ receptors, thereby mediating the inhibitory effects of leptin on appetite (Jørgensen et al., 2007). In addition, amylin, a peptide secreted from the pancreas following a meal or in response to oral glucose ingestion, acts as a satiation factor, and has been found to be overexpressed in genetically obese animals due to receptor insensitivity (Morley et al., 1995). Oxyntomodulin (OXM) is another peptide released from the gut following food intake and binds to both glucagon-like peptide-1 receptor (GLP1R) and the glucagon receptor; both systemic and brain-specific injection of (OXM) in rats reduces food intake and weight gain (Cohen et al., 2003). Interestingly, some orphan GPCRs, including GPR61 and GPR82, appear to be involved in regulating food intake; loss of GPR61 and GPR82 in mice resulted in hyperphagia and lower food intake, respectively (Engel et al., 2011; Nambu et al., 2011).

Gastrointestinal (GI) motility, digestion, and nutrient sensing also involve GPCRs. Taste receptors belong to the T1R and T2R families of GPCRs; ligand (amino acid) binding triggers signal transduction and neurotransmitter release, and taste receptors can also be found in certain regions of the gut to regulate gastric inhibitory polypeptide (GIP) and GLP-1 stimulated insulin secretion (Chaudhari and Roper, 2010; Efeyan, Comb and Sabatini, 2015). The neuropeptide cholecystokinin (CCK) can slow gastric emptying (via induction of acetylcholine release from the GI tract) (Paton et al., 1971), and additionally acts as an appetite suppressant (Crawley and Corwin, 1994). In addition, endogenous opioids of three known classes have been found throughout the gastrointestinal tract (Kromer, 1988), and exogenous opioids are well known to depress GI motility (Schaumann, 1955); localization of peptide YY (PYY), a ligand for neuropeptide Y receptors, also decreases intestinal blood flow and motility (Lundberg et al., 1982). Overall, this non-exhaustive list of GPCRs and their related signalling
pathways demonstrates the fundamental role of these receptors in mediating energy metabolism.

1.11 Altered G protein signalling in lean and obese phenotypes

Alterations in G protein signalling can lead to both lean and obese phenotypes. Loss of melanocortin 4 signalling results in severe obesity that is associated with hyperphagia, hyperinsulinemia, and hyperglycemia (Huszar et al., 1997); a triple knockout of β1/2/3-adrenergic receptors in mice led to weight gain on a standard chow diet, and the development of massive high fat diet induced obesity, which was attributed to a failure to initiate diet-induced thermogenesis (Bachman et al., 2002). Conversely, mice without Gβ5, a G protein β subunit that dimerizes with RGS proteins of the R7 family instead of Gγ, displayed a lean phenotype, were resistant to a high-fat diet, and demonstrated increased thermogenesis (Wang et al., 2011). Mice lacking the M3 muscarinic acetylcholine receptor are lean and hypophagic, indicating M3 receptor-mediated facilitation of food intake (Yamada et al., 2001). Moreover, loss of the neuropeptide melanin-concentrating hormone (MCH), which normally promotes feeding, led to reduced body weight due to hypophagia (Shimada et al., 1998). In addition, full-body RGS4 (Grillet et al., 2005) and RGS5 (Cho et al., 2008) knockout mice weight significantly less than WT counterparts, although lack of RGS5 has also been reported to exacerbate obesity, inflammation, hepatic steatosis, and insulin resistance (Deng et al., 2012). Rgs2−/− mice are also lean and display a hyperphagic phenotype, which will be subsequently discussed in detail.

1.12 Skeletal tissue is a major site of energy metabolism via thyroid hormone (TH) regulation

Thyroid hormone (TH) signalling is critical for numerous biological functions including development and growth, energy expenditure, and thermogenesis. TH primarily acts through nuclear-receptor mediated stimulation or inhibition of gene transcription (Yen, 2001; Mebis et
This activity is mainly mediated by the alpha isoform of the thyroid hormone receptor in skeletal muscle. Thyroid hormone receptors in the unliganded state are usually bound to TH-responsive elements in various genes along with other proteins, leading to active repression of gene transcription (Yen, 2001); upon ligand binding, repression of the target gene is lifted and transcription is stimulated by co-activator recruitment to the complex (Brent, 2012). In skeletal tissue, proteins which are transcriptionally regulated by TH include SERCA1A (Simonides et al., 1996), SERCA2A (Hartong et al., 1994), GLUT-4 (glucose transporter) (Zorzano, Palacin and Guma, 2005), UCP3 (muscle-specific) (Solanes et al., 2005), malic enzyme (ME1) (Desvergne, Petty and Nikodem, 1991), and glycerol – 3-phosphate dehydrogenase (Dümler, Müller and Seitz, 1991). In addition to modifying the glycolytic and oxidative capacities of skeletal tissue, TH affects mitochondrial capacity via transcription of the gene for PGC1α, a regulator of mitochondrial biogenesis (Weitzel, Iwen and Seitz, 2003).

Resting metabolic rates in almost all tissues depend on thyroid hormone availability. TH has been shown to increases the rates of muscle contraction and relaxation, which is related to changes in the expression of proteins involved in intracellular Ca\(^{2+}\) flux (e.g. SERCA isoforms) (Salvatore et al., 2013). By promoting a shift from slow type I fibre phenotypes to the faster type II skeletal fibres, TH induces the increased expression of myosin and SERCA, leading to greater energy turnover and the generation of heat during activity (Thomas, Auwerx and Schoonjans, 2008). In skeletal muscle, TH signalling can alter the metabolic rate by uncoupling ATP synthesis in the mitochondria or by decreasing metabolic efficiency; a potential mechanism through which energy is “wasted” in the form of heat dissipation includes decreasing ATP synthesis efficiency via proton leak through skeletal tissue specific mitochondrial uncoupling protein 3 (UCP3) (de Lange et al., 2001). Since skeletal tissue comprises approximately 40-50% of total body mass, it is the largest single contributor to the metabolic resting state of an organism, as well as towards heat generation (Thomas, Auwerx and Schoonjans, 2008).
1.13 TH regulation of metabolism and glucose homeostasis requires deiodinase type 2 (DIO2) for T4-T3 conversion

In order for thyroid hormone to regulate various metabolic processes, it must be converted from its inactive prohormone form T4/thyroxine (3,5,3',5'-tetraiodothyronine) to the active thyroid hormone/T3 (3,5,3'-triiodothyronine, T3). Thyroid hormone signalling in individual tissues can change even as serum T4 concentrations remain normal due to local activation or inactivation of thyroid hormone, the underlying mechanism of which is deiodination by the intracellular iodothyronine deiodinases type I, II, and III (DIO1, DIO2, and DIO3) (St. Germain and Galton, 1997; Bianco et al., 2002). DIO1, 2, and 3 regulate thyroid hormone activity via removal of iodine moieties from T4 (2); DIO2 converts T4 to T3, whereas DIO3 inactivates T3 (DIO1 function remains to be clarified) (Wondisford, 2003; Bianco and Kim, 2006). Thus, the deiodinases, and specifically DIO2, are critical determinants of intercellular T3 levels.

Glucose uptake into mammalian cells is mediated via the tissue specific transport proteins GLUT1-4, a process which is often a rate-limiting step in tissue glucose metabolism (Torrance et al., 1997). In skeletal muscle, insulin-sensitive GLUT-4 (or SLC2A4) is the main isoform expressed, and functions to rapidly remove circulating glucose from the blood following a meal; indeed, skeletal muscle accounts for more than 85% of whole body insulin-stimulated glucose uptake (Torrance et al., 1997). As previously mentioned, GLUT-4 protein and mRNA expression are regulated by TH (specifically T3); in conjunction, GLUT-4 translocation to the cell surface is an insulin-dependent event, and normal muscle responsiveness to insulin has been shown to require the DIO2-mediated conversion of T4 to T3 (Mills et al., 1987; Zorzano, Palacin and Guma, 2005; Grozovsky et al., 2009). The importance of DIO2 regulation of T3 production and insulin action can be seen through in vitro and animal models. Cultured myotubes lacking DIO2 have decreased phosphorylated Akt (a downstream effector of insulin) (Grozovsky et al., 2009), and mice with targeted deletion of Dio2 are insulin resistant and susceptible to diet-induced obesity (Marsili et al., 2011).
1.14 Role for RGS2 in metabolic pathways

Human polymorphisms that result in the over- or under-expression of RGS2 point to the clinical relevance of RGS2 in the context of metabolism. A common polymorphism in the 3’ untranslated region of RGS2 has been shown to decrease RGS2 expression, and is considered to be a genetic marker that can predict male predisposition to weight gain and obesity (Sartori et al., 2008). Conversely, a C to G substitution in the RGS2 promoter region has been shown to increase RGS2 expression in adipocytes and is associated with the metabolic syndrome (Freson et al., 2007). RGS2 has been shown to have various effects on energy storage and usage; *in vitro* studies have demonstrated the role of RGS2 in mediating GLUT-4 (insulin-responsive glucose transporter) translocation in adipocytes (Imamura et al., 1999), as well as GIP (glucose-dependent insulinotropic polypeptide)-induced insulin release from pancreatic β cells (Tseng and Zhang, 1998). Thus, RGS2 may be involved in glucose entrance in adipocytes. RGS2 may also regulate metabolism via inhibitory effects on thyroid stimulating hormone receptor (TSHR)-induced Gq signal transduction; the TSHR ligand TSH is a pituitary hormone which stimulates the thyroid gland to produce T4 (Eszlinger et al., 2004). RGS2 also appears to promote the differentiation of adipocytes and expression of adipogenic markers, and is transiently and rapidly upregulated in differentiating mouse preadipocyte cells (Imagawa, Tsuchiya and Nishihara, 1999; Nishizuka et al., 2001). Other energy-dependent processes affected by RGS2 include the regulation of protein synthesis (Nguyen et al., 2009; Chidiac et al., 2014), as well as ATP hydrolysis by PMCA (plasma membrane Ca\(^{2+}\) ATPase) and SERCA (sarco/endoplasmic reticulum Ca\(^{2+}\)) pumps (Wang et al., 2004), which altogether suggests an important role for RGS2 in body metabolism.

1.15 *Rgs2*\(^{-/-}\) mice display a lean phenotype and are hyperphagic

*Rgs2*\(^{-/-}\) mice, which are a well-established strain, demonstrate normal circadian activity and motor function; phenotypes include increased anxiety, decreased male aggression, reduced T cell proliferation and heightened sensitivity to TAC-induced cardiac hypertrophy, as well as mild to moderate levels of hypertension. (Oliveira-Dos-Santos et al., 2000; Heximer et al., 2003; Takimoto et al., 2009). Our lab has previously reported a novel phenotype in *Rgs2*\(^{-/-}\)
mice, such that they are resistant to age-related, normal body weight gain until their end-of-life (~24 months-old) (Nunn et al., 2011). In addition to decreased body weights, Rgs2^−/− mice are hyperphagic, display increased insulin sensitivity, decreased serum lipids, smaller brown and white adipose tissue deposits, and smaller adipocytes. Since RGS2 appears to promote adipocyte differentiation (Nishizuka et al., 2001), it would follow that its loss would result in impaired adipocyte function. Surprisingly, Rgs2^−/− mice were not resistant to high fat diet-induced obesity, and gained weight at similar rates to WT controls. Furthermore, the increased insulin sensitivity observed under *ad libitum* diet conditions was abrogated by the high fat diet (Nunn et al., 2011). Our observations suggest that even though adipocytes in Rgs2^−/− animals are significantly smaller compared to WTs, lipid metabolism may not be impaired, and that it is rather carbohydrate or protein metabolism which is altered. Given that our characterization of the lean metabolic phenotype mainly focused on adipocyte-related functions, exploring potential alterations in Rgs2^−/− skeletal tissue, a major metabolic contributor, could lead to a more in-depth understanding of the role of RGS2 in energy metabolism.

1.16 Diet-induced models of weight gain and loss

1.16.1 High-fat diets

Surrogate animal models are important for studying the molecular aspects of obesity and its pathophysiological effects, as patient complications can often take years to develop (Wang and Liao, 2012). Since their introduction three decades ago (Surwit et al., 1988), high-fat diets have been used extensively to study the pathophysiologies of weight gain (*e.g.* glucose intolerance and type 2 diabetes) and for the development of novel obesity treatments (Ahrén et al., 2000; Reimer, Holst and Ahrén, 2002). Wild-type animals on a high fat diet (particularly the C57/Bl/6 strain) gradually gain weight over time, and progressively develop increased hyperinsulinemia, which is indicative of worsening insulin resistance (Winzell and Ahrén, 2004). While standard chow has an approximate composition of less than ~10% kcal from fat, high fat diets can range from ~40% to ~60% kcal from fat content (Wang and Liao, 2012). Numerous high-fat studies use either lard (animal fat) or shortening (hydrogenated vegetable...
oil) (Silva et al., 2006; Lionetti et al., 2014; Fujiwara et al., 2015; Magri et al., 2015); vegetable shortening may also differ in their fatty acid composition depending on whether a partially hydrogenated soybean/palm oil or a partially hydrogenated soybean/cottonseed oil formulation is used (Kubant et al., 2015). Recent studies have found that lard-based high-fat diets increase weight gain and the development of obesity and insulin resistance to a greater extent than hydrogenated vegetable shortening diets (Kubant et al., 2015); this highlights the importance of identifying the fatty acid composition of diets used to examine the role of dietary fats.

1.16.2 Dietary restriction

Diet restriction models have most often been used in the context of aging and metabolism (Weindruch and Walford, 1982; Hursting et al., 2003; Bordone and Guarente, 2005); 30-50% food intake restrictions on ad libitum fed rats and mice have been shown to markedly increase longevity and delay the onset of age-associated diseases (Masoro, 2000). Intermittent fasting (e.g. 24 hours with/without access to food) has been shown to reduce serum glucose and insulin levels as well as neuronal resistance to injury (Anson et al., 2003). Chronic diet restriction, in which a group of animals is taken down to a set percentage of food intake (e.g. 70% of ad libitum intake), also has purported beneficial effects (McCarter, Masoro and Yu, 1985) Both chronic and acute (fasting) diet restriction conditions can be used to characterize mice with metabolic phenotypes; altered responses to dietary challenges compared to WT mice may reveal whether proteins or genes of interest are required for a normal response to caloric restriction (Boily et al., 2008).

1.17 Summary

Overall, RGS2 appears to play multiple roles in both cardiovascular disease and metabolism. The following chapters will explore novel ways in which the in vivo expression or lack of RGS2 impacts cardiovascular health and metabolism.
1.18 References


41


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2  HYPOTHESES AND SPECIFIC AIMS
2.1 Specific Aim 1

To determine if RGS2\textsuperscript{eb} is cardioprotective against experimentally-induced cardiac hypertrophy

Based on our previous studies and the findings of others, RGS2 is known to play a protective role against cardiac hypertrophy (Chidiac et al. 2014). In addition, loss of RGS2 in cell and animals studies is characterized by increased protein synthesis and severe cardiac hypertrophy (Takimoto et al. 2009). In conjunction with our previously reported cell data, \textit{in vivo} studies would provide a more comprehensive view of the antihypertrophic and possible cardioprotective effects of RGS2\textsuperscript{eb} under both pathological and physiological conditions.

Our \textit{principle goal} is to study the effects of RGS2\textsuperscript{eb} in animal models, and thus establish its \textit{in vivo} importance as a protective mechanism against cardiac hypertrophy and heart failure. Our \textit{specific objectives} are:

\begin{itemize}
  \item \textit{i)} To determine whether expression of RGS2\textsuperscript{eb} protects against experimentally-induced cardiac hypertrophy (\textbf{Study 1A}),
  \item \textit{ii)} To perform loss of function studies to determine \textit{in vivo} mechanisms of RGS2\textsuperscript{eb} cardioprotection (\textbf{Study 1B}),
\end{itemize}

\textbf{2.1.1 Study 1A: Protective effect of RGS2\textsuperscript{eb} in animal models of cardiac hypertrophy}

Regardless of the initial stimulus, \textit{de novo} protein synthesis is required for cardiomyocyte hypertrophy. We have previously shown that expression of RGS2\textsuperscript{eb} in cultured neonatal rat cardiomyocytes can inhibit hypertrophy via the attenuation of mRNA translation (Chidiac et al. 2014). However, it is unknown whether the protective effect of RGS2\textsuperscript{eb} extends to whole animals with experimentally-induced cardiac hypertrophy.
The objectives of this study are:

1) To determine whether animals with targeted myocardial expression of RGS2<sup>eb</sup> show resistance to the development of pressure overload induced cardiac hypertrophy in comparison to wild-type controls.

2) To determine whether animals with targeted myocardial expression of RGS2<sup>eb</sup> demonstrate improved cardiovascular function in an experimental pressure overload model in comparison to wild-type controls.

We hypothesize that due to the ability of RGS2<sup>eb</sup> to inhibit protein synthesis, transgenic mice will show decreased severity of cardiac hypertrophy and potentially improved cardiac function in comparison to wild type mice.

2.1.2 Study 1B: Rescue effect of RGS2<sup>eb</sup> in RGS2 knockout animals

Rgs2<sup>-/-</sup> mice are characterized by severe cardiac hypertrophy and heart failure following experimentally induced pressure overload (Takimoto et al. 2009). This demonstrates the protective cardiac effects of RGS2. However, the degree to which the inhibition of global protein synthesis by RGS2<sup>eb</sup> contributes to this effect is unknown. In addition, the possible protective effect of RGS2<sup>eb</sup> against drug-induced hypertrophy has not been explored.

The objectives of this study are:

1) To determine whether expression of RGS2<sup>eb</sup> in Rgs2<sup>-/-</sup> mice can compensate for the loss of the full length RGS2 protein in comparison to null RGS2 mice following experimentally-induced pressure overload.

2) To characterize the effects of drug-induced hypertrophy in Rgs2<sup>-/-</sup> with cardiac-specific expression of RGS2<sup>eb</sup>.

We hypothesize that via its inhibition of protein synthesis, RGS2<sup>eb</sup> will, to some extent, decrease the severity of cardiac hypertrophy and heart failure resulting from experimentally
induced pressure overload or drug-induced hypertension and hypertrophy, thereby compensating for the absence of full-length RGS2.

2.2 Specific Aim 2

To determine the metabolic effects of RGS2 under multiple diet modalities

Aside from an increased sensitivity to pressure-induced cardiac hypertrophy (Takimoto et al. 2009), $Rgs2^{-/-}$ mice display several additional phenotypes, including decreased male aggression, increased anxiety, elevated blood pressure, and reduced T-cell proliferation (Oliveira-Dos-Santos et al. 2000; Heximer et al. 2003). Our lab has previously determined that $Rgs2^{-/-}$ mice exhibit a weight-related phenotype, such that they fail to develop the gains in body fat that normally occur in freely-feeding laboratory mice (Nunn et al. 2011). We demonstrated that resistance to age-related body weight gain was accompanied by smaller adipose tissue deposits and adipocytes, lower levels of serum lipids, and increased insulin sensitivity; these data suggest that RGS2 may play a novel role in the regulation of fat production and energy metabolism. $Rgs2^{-/-}$ mice were found to be slightly hyperphagic over time in comparison to WT controls, and did not have impaired nutrient absorption. However, measurements of resting $O_2$ consumption ($VO_2$) using a basic open flow respirometry system suggested a lower metabolic rate in $Rgs2^{-/-}$ mice compared to WT animals (Nunn et al. 2010). Thus, it would seem that although $Rgs2^{-/-}$ mice expend less energy at rest, eat more, and absorb the same amount of nutrients, they still weigh less in comparison to WT controls. Furthermore, we were not able in our previous studies to detect any increase in resting $VO_2$ in $Rgs2^{-/-}$ mice using a basic open flow respirometry system. Our previous studies have provided contradictory evidence for the reduction of weight gain in RGS2 KO mice. To resolve this seeming paradox, extended and acute measurements of metabolic indicators may provide a more comprehensive understanding of RGS2 and its role in metabolism and weight gain.
Our principle goal is to understand the effects of loss of RGS2 on body metabolism, and thus establish its in vivo role. Our specific objectives are:

1) To determine baseline metabolic characteristics of Rgs2<sup>−/−</sup> mice in comparison to age-matched WT controls (Study 2A),

2) To perform diet restriction studies to further characterize the Rgs2<sup>−/−</sup> metabolic phenotype (Studies 2B and 2C)

2.2.1 Study 2A: Baseline characterization of the Rgs2<sup>−/−</sup> metabolic phenotype

In comparison to WT controls, Rgs2<sup>−/−</sup> mice are resistant to age-related weight gain. However, previous measurements of metabolic activity have offered paradoxical evidence on the potential reasons for this lean phenotype. The objective of this study is:

1) To determine whether metabolism in Rgs2<sup>−/−</sup> is altered, particularly during the dark phase of a 12 hour light-dark cycle in comparison to WT controls, as measured via Comprehensive Laboratory Animal Monitoring System (CLAMS) metabolic cages

We hypothesize that Rgs2<sup>−/−</sup> will display increased metabolism as demonstrated by various metabolic and biological indices in comparison to WT controls.

2.2.2 Study 2B: Chronic diet restriction model

We have previously shown that while Rgs2<sup>−/−</sup> mice are resistant to age-related weight gain on a standard rodent diet (Teklad Global 18% Protein Rodent Diet), they still gain at least as much weight as WT mice when placed on a high fat diet. However, it is currently unknown how Rgs2<sup>−/−</sup> mice would respond metabolically to a diet restriction. The objective of this study is:

1) To determine whether metabolism in Rgs2<sup>−/−</sup> mice is altered in response to dietary restriction in comparison to WT controls.

We hypothesize that Rgs2<sup>−/−</sup> mice will display greater metabolism than their counterpart WT controls, as demonstrated by various metabolic and biological indices.
2.2.3 Study 2C: Acute diet restriction model

It is also currently unknown how Rgs2\textsuperscript{-/-} mice would respond metabolically to acute dietary restriction (\textit{i.e.} fasting conditions); this may also potentially offer insight into the resistance of Rgs2\textsuperscript{-/-} mice to age related weight gain. The \textit{objective} of this study is:

\begin{itemize}
  \item[i)] To determine whether Rgs2\textsuperscript{-/-} metabolism is altered after acute fasting in comparison to WT controls.
\end{itemize}

\textit{We hypothesize} that Rgs2\textsuperscript{-/-} mice will display greater metabolism under acute caloric restriction than corresponding WT controls.
2.3 References


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3 CARDIOMYOCYTE SPECIFIC OVEREXPRESSION OF A 37 AMINO ACID DOMAIN OF REGULATOR OF G PROTEIN SIGNALLING 2 INHIBITS CARDIAC HYPERTROPHY AND IMPROVES FUNCTION IN RESPONSE TO PRESSURE OVERLOAD IN MICE

3.1 Introduction

Pathological cardiac hypertrophy is a maladaptive growth response of the heart to a variety of disease stimuli. Induced by factors such as hypertension or valvular diseases, prolonged pathological hypertrophy has been associated with an increased risk of sudden death, as well as myocardial infarctions and arrhythmias (Levy et al., 1990; Weber and Brilla, 1991; Heineke and Molkentin, 2006). Moreover, maladaptive hypertrophy is a major risk factor for heart failure (Heineke and Molkentin, 2006). Given the high mortality rates following heart failure diagnoses and the current lack of a cure, reducing risk factors such as pathological cardiac hypertrophy may prove therapeutically beneficial.

G protein-coupled receptors (GPCRs) which signal via heterotrimeric G_αq and G_αs proteins are well established as critical players in the induction of pathological hypertrophy (Dorn and Force, 2005; Heineke and Molkentin, 2006; Hendriks-Balk et al., 2008). Clinically effective treatments for heart failure, such as angiotensin II converting enzyme (ACE) inhibitors and beta-adrenergic receptor antagonists, demonstrate the effectiveness of targeting G_q- and G_s-coupled receptors (Campbell et al., 2001). However, the effectiveness of these drugs is limited to slowing, rather than reversing, the progression of heart failure. Regulator of G protein signalling 2 (RGS2) is a GTPase accelerating protein (GAP) found ubiquitously throughout the body. RGS2 selectively inhibits G_q- and G_s-mediated signalling (some effects on G_i/o signalling have also been reported (Anger et al., 2007; Chakir et al., 2011), thus making it an important target in the study of cardiovascular disease (Heximer et al., 1997, 1999; Roy, Baragli, et al., 2006). Studies in vivo and in cardiomyocytes have shown that hypertrophy caused by prolonged G_q-coupled receptor stimulation, such as that induced by phenylephrine, can be blocked by the overexpression of RGS2 (Roy, Nunn, et al., 2006; Zou et al., 2006). A similar effect has been seen against G_s-mediated cardiomyocyte hypertrophy induced by isoproterenol (Nunn et al., 2010). These observations suggest that RGS2 plays an important role in the regulation of hypertrophy; This has been further demonstrated in knockout animal studies. In RGS2 null mice, experimentally induced pressure overload causes marked hypertrophy, heart failure, and death, as well as increased expression of cardiac fetal genes...
(Takimoto et al., 2009). Thus, RGS2 is an essential element in the prevention of pathological cardiac hypertrophy.

While the G protein inhibitory effects of RGS2 are well established, studies have also shown that RGS2 can bind to and regulate other targets, including TRPV6 calcium channels and tubulin (Wang et al., 2005; Heo et al., 2006; Schoeber et al., 2006). We have previously shown that RGS2 can bind to the ε subunit of eukaryotic initiation factor 2B (eIF2B), a component of the rate-limiting step of the initiation of mRNA translation (Nguyen et al., 2009). By interacting with eIF2Bε, RGS2 limits GDP dissociation on eukaryotic initiation factor 2 (eIF2), which ultimately leads to the attenuation of *de novo* protein synthesis. This property of RGS2 has been mapped to a 37 amino acid domain (residues 79-115) termed RGS2ε, that is homologous to a region in the β subunit of eIF2 (Nguyen et al., 2009).

Since the heart is considered to be a post-mitotic organ (Anversa et al., 2007), hypertrophic growth is thought to be dependent on the enlargement of a pre-existing cardiomyocyte population rather than cell division (Carvalho, 2010). Therefore, regardless of the initial stimuli and receptors involved, all hypertrophic signals will ultimately result in increased mRNA translation and *de novo* protein synthesis. Our previous studies have shown that RGS2ε expression in cultured neonatal cardiomyocytes is able to inhibit both protein synthesis and agonist induced hypertrophy at levels comparable to full-length RGS2 (Chidiac et al., 2014). Based on these previous findings, we hypothesized that the *in vivo* expression of RGS2ε in the murine heart could attenuate the development of pathological cardiac hypertrophy.

To determine whether RGS2ε could act as an *in vivo* anti-hypertrophic agent, we developed a line of transgenic mice with cardiomyocyte-specific overexpression of RGS2ε, and used transverse aortic constriction (TAC) to induce pressure overload on the heart. Here we report that following 4 weeks of aortic constriction, RGS2ε transgenic mice were protected not only from pressure overload-induced cardiac hypertrophy, but were able to maintain heart function at significantly improved levels compared to WT TAC mice. Moreover, reactivation of the “fetal gene program”, an indicator of hypertrophy and heart failure, was suppressed. Notably, cardiomyocyte size was decreased in RGS2ε TG compared to WT TAC controls, further supporting our earlier *in vitro* studies which showed RGS2ε inhibition of *de novo* protein
synthesis. Together, these findings suggest that in addition to its G-protein inhibitory actions, the RGS2\textsuperscript{eb} region may be contributing to the cardioprotective effects of full-length RGS2 \textit{in vivo} via the inhibition of \textit{de novo} protein synthesis.

### 3.2 Methods

#### 3.2.1 Generation of myosin heavy chain promoter (MHC) – RGS2\textsuperscript{eb} transgenic mice

The RGS2\textsuperscript{eb} gene was targeted to the heart using the mouse α-MHC promoter (kindly provided by Jeffrey Robbins, Cincinnati Children’s Hospital Medical Center)(Subramaniam \textit{et al.}, 1991). Transgenic mice were generated in the FVB background (London Regional Transgenic and Gene Targeting Facility) and identified by polymerase chain reaction (PCR). Briefly, ear biopsies were taken from three-week old mice and purified for genomic DNA using the QIAquick PCR purification kit (Qiagen). PCR was performed using DreamTaq Green PCR Master Mix (Thermo Scientific). Primers for detection of transgenic mice (CTGCTAGCCAGCAAATATGGTC forward, CCTACAGGTTGTCTTCCCCAACT reverse) and control primers for endogenous RGS2 expression (CCGAGTTCTGTGAAGAAAACATTG forward, ATGCTACATGAGAACCCAGGAGTCC reverse) were designed using the OligoPerfect Designer (ThermoFisher), OligoCalc (Kibbe, 2007), and Primer-BLAST (NCBI) programs, resulting in a 293 bp and 342 bp fragment, respectively. RGS2\textsuperscript{eb} TG transgenic mice were back-crossed with C57Bl/6 mice (Charles River) for at least seven generations before animal experiments were performed. Animals were maintained in accordance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. These studies were approved by the Animal Care Committee at the University of Western Ontario, and complied with the guidelines of the Canadian Council on Animal Care.
3.2.2 Transverse aortic constriction (TAC)

TAC was used to induce pressure overload on the hearts of 12 week old male C57BL/6 wild-type mice and RGS2<sup>eb</sup> TG littermates. Mice were anaesthetized with a ketamine (50 mg/kg) and xylazine (12.5 mg/kg) cocktail intramuscularly, intubated, and ventilated with a respirator (SAR-830, CWE, Ardmore, PA, USA). To access the chest cavity, thoracotomy was performed at the second intercostal space under a surgical microscope (Rockman <i>et al.</i>, 1991). A 6-0 silk suture was placed between the brachiocephalic and left carotid arteries. Two knots were tied against a 25-gauge blunt needle placed parallel to the transverse aorta. The needle was removed immediately after the second tied knot followed by closure of the chest. Control WT and RGS2<sup>eb</sup> TG mice were subjected to sham operations without aortic constriction.

3.2.3 Assessment of cardiac function

Hemodynamic measurements were performed as previously described (Detombe <i>et al.</i>, 2008; Pacher <i>et al.</i>, 2008): four weeks post-surgery, mice were again anaesthetized with a ketamine and xylazine cocktail and ventilated. A Millar micro-tip pressure catheter was inserted into the left carotid artery to assess carotid artery pressure, followed by removal of the catheter and insertion into the right carotid artery for pressure readings, and then advanced into the left ventricle (LV) to measure LV pressures, volumes, and heart rate at steady state and during transient preload reduction via mechanical occlusion of the inferior vena cava. All data were recorded using a PowerLab data acquisition system and analysed by LabChart 7.0 (ADInstruments) and PVAN 3.4 software (Millar).

3.2.4 Heart weight/body weight ratios

Upon completion of hemodynamic recordings, mice were immediately euthanized via a 10% KCl injection into the left jugular vein to ensure cardiac arrest in the diastolic state. Hearts were excised, weighed after removal of the atria, then cut transversely into three equal sections, with the middle section reserved for histological analysis, and the remaining tissue sections stored at -80°C for subsequent RNA isolation.
3.2.5 Histological analysis

Heart samples were fixed in 4% paraformaldehyde overnight at 4°C, dehydrated, and paraffin embedded. Samples were sectioned into 5 µm thick slices with a Leica RM2255 microtome, mounted onto positively charged slides, and then stained with haematoxylin and eosin. Images of left ventricles were captured at 400X magnification with a Zeiss Observer D1 microscope using AxioVision 4.7 software (Zeiss) for cardiomyocyte size measurements and immunohistochemical imaging; images of the LV for wall thickness measurements were captured at 100X magnification.

3.2.6 Cardiomyocyte Cell Size and LV wall thickness

Haematoxylin and eosin stained tissue sections were used to determine left ventricular cardiomyocyte cell size and wall thickness of LV free walls and septum. The size of an individual cardiomyocyte was determined by measuring its cross-sectional area. All cardiomyocytes with a well-defined border were manually outlined and then filled in the open source GNU Image Manipulation Program (GIMP). Images were then opened in the image processing program ImageJ and analyzed after setting the threshold. Cardiomyocytes with a circularity ratio of ≥1.2 were excluded to eliminate cells sectioned tangentially (Helms et al., 2010). Areas of at least 33 cells per animal were measured, and were scored blind to surgeries and strain.

Heart wall thickness of the LV free wall was measured using AxioVision 4.7 software (Zeiss) in three distinct areas within and between the anterior and posterior regions of the free wall; three measurements were taken from each area for a total of 9 averaged measurements per sample. For the septal wall, single measurements were taken from three distinct areas of the wall and averaged for each sample.

3.2.7 Immunohistochemistry

Immunohistochemical staining was performed on 5 µm thick paraffin heart sections. Samples underwent a deparaffinisation process and antigen retrieval was carried out in sodium citrate buffer (pH 6.0) at 92°C using a BP-111 laboratory microwave (Microwave Research and
Applications). Sections were incubated with primary antibody anti-6X His tag-ChIP Grade (Abcam) overnight followed by biotinylated secondary antibody, and signal was detected using avidin-biotin complex (ImmunoCruz ABC staining kit, Santa Cruz). Diaminobenzidine (DAB) substrate solution was used for antigen visualization with haematoxylin as a counterstain. Following staining, the LV free wall was imaged in three distinct locations within and between the anterior and posterior walls. Slides were imaged at 400X magnification with a Zeiss Observer D1 microscope using AxioVision 4.7 software. To ensure accurate comparisons of antigen visualization, sections from WT and RGS2\textsuperscript{eb} TG genotypes were stained simultaneously and all images were captured using the same microscopic parameters.

### 3.2.8 Dot blotting

Using mechanical disruption (Sonic Dismembrator Model 100, Fisher Scientific), LV heart and kidney tissues from RGS2\textsuperscript{eb} TG and WT mice were lysed in 20mM, pH 7.5 Tris-HCl buffer containing cOmplete Mini protease inhibitor cocktail (Sigma). Samples were centrifuged at 100,000 \( g \) for 1 hour at 4°C in an Optima TLX micro-ultracentrifuge (Beckman Coulter), and supernatants were kept for experiments. Following protein concentration determination by Bradford assay, equal amounts of sample protein were spotted directly on to Amersham Protran 0.2 µm pore size nitrocellulose membrane (GE Healthcare Life Sciences). Purified histidine-tagged RGS-16 (His-RGS16) was also dotted on to membranes to serve as a positive control. Dried membranes were blocked in 5% milk-TBST, followed by overnight incubation in 1:2000 ChIP grade anti-6X Histidine primary antibody (Abcam) and then 1:5000 goat anti-rabbit secondary antibody HRP conjugate (Invitrogen). Signal was detected using SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific) and imaged with the Versadoc MP 5000 system and Quantity One software (Bio-Rad).

### 3.2.9 Quantitative reverse transcriptase qPCR

Total RNA was isolated from heart tissue using the TriZol (Invitrogen) extraction method. Reverse transcription reaction was performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Invitrogen). SensiFAST SYBR No-ROX Kit mastermix (FroggaBio) was used for real-time thermal cycling. Primers for RGS2
(TGGGATTATGTGGCCTTAGC forward, AAGAACGTCAACACCCTTGC reverse), hGH (TGGGAAGACAACCTGTAGGG forward, AATCGCTTGAACCCAGGAG reverse), β-MHC (CTGAGACGGAGAATGGCAAGAC forward, ACTTGTTAGGGGTTGACGGTGAC reverse), ANP (ATTCCTGAGACGTCCCTTTT forward, CATTCTCCATCCACAGCTCCT reverse), and BNP (TGGGAATTAGCTGTGAGAG forward, TTTGGGTGTTCTTTTGTGAGG reverse) were designed using the OligoPerfect Designer (ThermoFisher), OligoCalc (Kibbe, 2007) and Primer-BLAST (NCBI) programs. Samples were amplified for 35 cycles using the Eppendorf Mastercycler Realplex Real-Time PCR machine. The mRNA quantity for each gene of interest was determined using standard curve analysis and normalized to 28S ribosomal expression.

### 3.2.10 Statistical analysis

All data were analyzed using GraphPad Prism 6.01 (GraphPad). All statistical analyses were performed using two-way ANOVA followed by Bonferroni post-tests and presented as mean ± SEM. Differences were considered significant at P<0.05.

### 3.3 Results

#### 3.3.1 Generation of transgenic mice with cardiomyocyte specific expression of RGS2^eb^ 

We previously demonstrated that the *in vitro* expression of RGS2^eb^ was sufficient to inhibit drug-induced hypertrophy in isolated neonatal rat cardiomyocytes (Chidiac et al., 2014). To elucidate the potential *in vivo* protective role of RGS2^eb^, we developed a novel strain of transgenic mice with targeted myocardial overexpression of polyhistidine-tagged RGS2^eb^ under the control of the α-myosin heavy chain promoter (Fig. 3.1A). Genotyping with primers for a portion of the hGH polyA region, which was only present in the transgenic sequence of RGS2^eb^ mice, was used to differentiate between RGS2^eb^ TG and wildtype mice. Both RGS2^eb^ TG and WT controls displayed a band at 342 bp indicating the presence of endogenous full-length RGS2 (Fig. 3.1B); Only RGS2^eb^ transgenic mice displayed an additional 293 bp band,
which indicated the presence of the hGH poly A region. Due to the short length of the RGS2\textsuperscript{eb} transgene and difficulty in immunoblotting (Chidiac \textit{et al.}, 2014), immunohistochemical staining, qPCR for RGS2 and the human growth hormone (hGH), as well as dot blot visualization for the polyhistidine tag was used to confirm expression of the RGS2\textsuperscript{eb} transgene. Following DAB visualization, WT mice showed an absence of staining for polyhistidine, whereas RGS2\textsuperscript{eb} TG mice displayed positive antigen staining (Fig. 3.1C). Endogenous RGS2 appears to be decreased in both WT and RGS2\textsuperscript{eb} TG mice following TAC (Fig. 3.1D), which has been previously demonstrated (Zhang \textit{et al.}, 2006); Expression of a region of hGH, which should only be present in transgenic mice, is significantly higher in RGS2\textsuperscript{eb} TG mice than in WT animals (Fig. 1E). In addition, LV heart tissues from RGS2\textsuperscript{eb} TG mice showed positive signal for polyhistidine when visualized on dot blots, whereas no signal was detected in non-cardiac tissue (\textit{i.e.} kidney) and LV samples from WT mice (Fig. 3.2F). Together, these results indicate successful generation of transgenic mice with cardiomyocyte specific overexpression of RGS2\textsuperscript{eb}.

3.3.2 Improved cardiac function in RGS2\textsuperscript{eb} transgenic mice following pressure overload

Transverse aortic constriction (TAC) was used to induce experimental pressure overload on wildtype and RGS2\textsuperscript{eb} TG hearts. Following 4 weeks of constriction, systolic pressure was significantly increased in the right carotid artery, but significantly reduced downstream of the TAC site in the left carotid artery in both WT and RGS2\textsuperscript{eb} TG mice (Fig. 3.3A and 3.3B). This resulted in a 61 mmHg difference in maximal systolic pressure between the left and right carotid arteries of WT sham and TAC mice, and a 65 mmHg difference between RGS2\textsuperscript{eb} TG sham and TAC mice (Fig. 3.3C). These data, as well as summary hemodynamic parameters in Table 3.1, confirm that pressure overload had indeed been induced upon TAC hearts. Increased vascular resistance was reflected by significantly higher end-systolic pressures in TAC mice; increased afterload as well as cardiac dilation or hypertrophy in TAC mice was also suggested by significant increases in the end systolic and end diastolic volumes (Table 3.1). Heart rate under anaesthesia was maintained at similar levels in all surgery groups (Fig. 3.3D). Following pressure-volume loop analysis, RGS2\textsuperscript{eb} TG mice demonstrated improved cardiac function as
measured by various systolic and diastolic indices. Summary data for contractility and relaxation (maximal elastance, ejection fraction, left ventricular systolic pressure, arterial elastance, LV $+dP/dt$, LV $-dP/dt$, and time constant tau) are provided in Figure 3. Notably, Millar catheter measurements of the maximal and minimal rates of pressure change in the left ventricle (LV $+dP/dt$ and LV $-dP/dt$, respectively), which are important indices of cardiac contractility, were significantly greater in RGS2$^{eb}$ TG TAC mice compared to WT TAC counterparts (Fig. 3.4F and 3.4G). In addition, WT TAC mice had significant increases in the Tau relaxation constant (Fig. 3.4H), indicating impairment of active properties of diastolic relaxation. These data suggest that RGS2$^{eb}$ TG mice demonstrated improved compensation following 4 weeks of TAC compared to WT TAC animals.

3.3.3 Cardiac hypertrophy is inhibited in RGS2$^{eb}$ transgenic mice following pressure overload

Following 4 weeks of TAC, heart weight/body weight (HWT/BWT) ratios, LV and septal wall thickness, and the cross-sectional areas of cardiomyocytes were used to evaluate the development of cardiac hypertrophy. Although WT TAC mice developed significant cardiac hypertrophy (as measured by HWT/BWT) compared to sham controls, RGS2$^{eb}$ TG mice displayed limited hypertrophic growth compared to WT TAC animals (Fig 3.5A). Some hypertrophy does still appear to have occurred, as both WT and RGS2$^{eb}$ TG mice had significantly increased LV free wall thickness compared to sham controls (Fig. 3.5B, 3.5F and Fig. 3.6A (representatives)); Septal wall thickness was not significantly changed in TAC surgery groups compared to sham controls (Fig 3.5D). In addition, RGS2$^{eb}$ TG TAC mice did not show marked changes in LV free wall cardiomyocyte size compared to TG sham controls, and were significantly smaller in size than cardiomyocytes from WT TAC mice (Fig. 3.5E and 3.6B (representative)). No changes were observed in measured cardiomyocytes from the septal wall (Fig. 3.5F). These data suggest that RGS2$^{eb}$ may confer a protective effect in cardiomyocytes against pressure-induced cardiomyocyte hypertrophy.
3.3.4 Expression of cardiac hypertrophy markers is suppressed in RGS2\textsuperscript{eb} transgenic mice

Induction of “fetal” cardiac genes is often a common feature in animal models of pathological hypertrophy and heart failure (Parker, Packer and Schneider, 1990; Razeghi \textit{et al.}, 2001). RNA was isolated from left ventricular tissue after 4 weeks of TAC or sham surgery, and qPCR was performed to determine expression levels of β-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP). WT mice which underwent TAC showed significantly increased levels of both β-MHC and ANP compared to sham controls (Fig. 3.7A and B). In contrast, β-MHC and ANP expression in RGS2\textsuperscript{eb} TG mice after TAC was comparable to sham levels (Fig. 3.7A and B). BNP expression demonstrated a similar trend to that seen with β-MHC and ANP, but this did not attain statistical significance between RGS2\textsuperscript{eb} TG and WT mice after TAC (Fig. 3.7C). Since the expression of fetal cardiac genes is postulated to be indicator of cardiac dysfunction, the absence of elevated levels in RGS2\textsuperscript{eb} TG mice after TAC suggests that the presence of RGS2\textsuperscript{eb} is cardioprotective in a pressure overload model of cardiac hypertrophy.
Figure 3.1. Generation of transgenic mice with cardiomyocyte specific expression of RGS2eb

(A) Construct used for the generation of mice with cardiomyocyte-specific overexpression of RGS2eb under the control of α-myosin heavy chain. (B) Genotyping for a region which includes the hGH PolyA sequence results in a positive band for RGS2eb TG mice and the absence of a band in WT controls (lanes 8 and 10), while genotyping for endogenous RGS2 resulted in bands for both RGS2eb TG and WT controls (lanes 1, 3, 5, 7, and 9). (C) Representative immunostaining in the left ventricle of WT mice shows an absence of polyhistidine staining, whereas RGS2eb TG mice display positive immunostaining for polyhistidine. (D) RGS2 and (E) hGH qPCR expression in WT and RGS2eb TG mice following sham or TAC surgery. Data represent means ± SEM, n = 5-6 per group. **P<0.01, and ***P<0.001 using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
Figure 3.2. Dot blot detection of histidine-tagged RGS2<sup>eb</sup>

Signal for polyhistidine was only detected in the LV tissue of RGS2<sup>eb</sup> TG mice and His-RGS16 positive controls, with no signal detected in non-cardiac tissue or WT mice.
Figure 3.3. Pressure overload model of cardiac hypertrophy

(A) Following 4 weeks of aortic constriction between the left and right carotid arteries, systolic pressure in the LCA was reduced while (B) systolic pressure in the RCA was significantly increased. (C) Change in systolic pressure between the RCA and LCA following 4 weeks TAC was significantly increased in both WT and RGS2\textsuperscript{eb} TG mice. (D) Heart rate remained at similar levels for all experimental and genotype groups. Data represent means ± SEM, n = 7-9 per group. *P<0.05 and ***P<0.001 using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
Table 3.1. Hemodynamic parameters in 4 week sham and TAC mice.

Data represent means ± SEM **P<0.05 and **P<0.001 vs. sham controls using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.

<table>
<thead>
<tr>
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<th>SHAM</th>
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<td>WT</td>
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<td>7-13</td>
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<tr>
<td>BWT (g)</td>
<td>29.3±1.0</td>
<td>30.7±1.0</td>
<td>30.1±0.6</td>
<td>28.1±1.0</td>
</tr>
<tr>
<td>ESP (mmHg)</td>
<td>98.2±6.9</td>
<td>73.3±14</td>
<td>134.1±8.1*</td>
<td>144.2±11**</td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>7.6±1.4</td>
<td>6.0±2.3</td>
<td>15.0±3.9</td>
<td>15.5±5.6</td>
</tr>
<tr>
<td>ESV (μL)</td>
<td>18.4±4.4</td>
<td>16.7±0.5</td>
<td>37.0±5.9*</td>
<td>39.3±6.5*</td>
</tr>
<tr>
<td>EDV (μL)</td>
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<td>25.9±2.3</td>
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<td>51.4±6.2*</td>
</tr>
<tr>
<td>SV (μL)</td>
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<td>18.5±2.6</td>
<td>14.1±2.1</td>
<td>15.7±2.7</td>
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<td>SW (mmHg*μL)</td>
<td>1456.9±207</td>
<td>1523.8±187</td>
<td>1661.9±207</td>
<td>1667.6±231</td>
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<tr>
<td>CO (μL/min)</td>
<td>7112.3±947</td>
<td>6517.8±907</td>
<td>6713.6±596</td>
<td>8152.1±1098</td>
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BWT, body weight; ESP, end systolic pressure; EDP, end diastolic pressure; ESV, end stroke volume; EDV, end diastolic volume; SV, stroke volume; SW, stroke work; CO, cardiac output
Figure 3.4. Indices of systolic and diastolic function

(A) Representative PV loops during preload reduction by inferior vena cava occlusion in sham (solid line) and 4 week TAC animals (dotted line). (B) Slope of left upper relation (maximal elastance [$E_{\text{max}}$]) reflected contractile function. (C) Ejection fraction was significantly reduced in WT but not RGS2$^{\text{eb}}$ TG mice following pressure overload, while (D) LV systolic pressure (LVSP) was significantly elevated following TAC in both WT and RGS2$^{\text{eb}}$ mice. (E) Effective arterial elastance ($E_a$; an index of total ventricular afterload) was also increased following TAC. (F) LV +dP/dt and (G) −dP/dt were significantly improved in RGS2$^{\text{eb}}$ mice following TAC, and (H) Tau relaxation time constant remained comparable to sham groups. Data represent means ± SEM, n = 6-9 per group. *P<0.05 and ***P<0.001 using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
Figure 3.5. Cardiac hypertrophy is reduced in RGS2\textsuperscript{eb} transgenic mice following pressure overload

(A) After 4 weeks of aortic constriction, cardiac hypertrophy in RGS2\textsuperscript{eb} TG mice was significantly less than WT TAC. (B) Representative whole hearts for WT and RGS2\textsuperscript{eb} TG mice following 4 weeks of sham or TAC surgery; (C) LV free wall thickness was increased in both TAC groups; (D) septal wall thickness did not increase. (E) Cardiomyocyte size was significantly reduced in RGS2\textsuperscript{eb} TG mice compared to WT TAC in the LV free wall, with (F) no noticeable changes to size in the septal wall.
Figure 3.6. LV cross sections and cardiomyocytes

A) Representative H&E staining of WT and RGS2\textsuperscript{eb} LV cross sections of WT and RGS2\textsuperscript{eb} TG cardiomyocytes following sham or TAC surgery. (B) Representative H&E staining of WT and RGS2\textsuperscript{eb} cardiomyocytes.
Figure 3.7. Markers of cardiac hypertrophy

(A) β-MHC expression was significantly increased in WT TAC but not RGS2\textsuperscript{ch} TG mice after 4 weeks of aortic constriction. (B) ANP expression was also significantly elevated in WT TAC mice, while RGS2\textsuperscript{ch} TG mice maintained near-sham levels of expression. (C) BNP expression. Data represent means ± SEM, n = 4-8 per group. *P<0.05, **P<0.01, and ***P<0.001 using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
3.4 Discussion

In order for maladaptive cardiac hypertrophy to occur, individual cardiomyocytes must increase in size; such growth requires increased global protein synthesis, the rate of which is controlled primarily at the initiation level (Sugden et al., 2009). Of particular importance is the heterotrimeric initiation factor eIF2, which is activated by the heteropentameric protein eIF2B (Abbott and Proud, 2004). Notably, eIF2B has been shown to play a critical role in the development of β-adrenergic receptor induced hypertrophy in cultured cardiomyocytes (Hardt et al., 2004). A major role for eIF2Bε in controlling cell size is also evident from its regulation by glycogen synthase kinase 3β (GSK3β), which constitutively phosphorylates eIF2Bε at serine 540 in intact cells, thereby inhibiting its GEF activity by up to 80% (Welsh et al., 1998).

In a cultured cardiomyocyte model, overexpression of a mutant eIF2Bε which could not be phosphorylated by GSK3β increased cell size and abolished the antihypertrophic effects of GSK3β, suggesting that eIF2Bε is a direct mediator of cardiac myocyte hypertrophy (Hardt et al., 2004). Importantly, further expression of a dominant-negative mutant of eIF2Bε inhibited isoproterenol-induced cardiac hypertrophy, indicating the critical role of eIF2Bε in protein synthesis and cell growth (Hardt et al., 2004).

We have previously shown that the cellular effects of eIF2Bε are blocked by RGS2εb as well as by full length RGS2 (Nunn et al., 2010; Chidiac et al., 2014). The goal of the present study was to determine whether the ability of RGS2 to inhibit protein synthesis and cardiomyocyte growth in vitro could be extended to an animal model of pathological cardiac hypertrophy. We therefore developed a mouse line with cardiomyocyte specific expression of RGS2εb, and examined the functional, histological, and biochemical consequences of experimental pressure overload in our transgenic mice and WT littermates. To the best of our knowledge, this is the first study to demonstrate that expression of a portion of RGS2, namely the RGS2εb binding domain, can reduce pathogenic growth without adversely affecting in vivo cardiac function. Although a random insertion effect cannot be entirely excluded, expression of this transgene inhibited TAC-induced increases in heart weight/body weight ratio (Fig. 3.5A) and expression of hypertrophy-related gene markers (Fig. 3.7), as well as associated functional loss (Fig. 3.4).
These results likely reflect the ability of RGS2<sup>eb</sup> to inhibit protein synthesis <i>in vivo</i>, and the consequent suppression of the hypertrophic response and cardioprotective effects.

Using qPCR, immunohistochemistry and dot blotting methods, we have demonstrated the cardiac-specific expression of RGS2<sup>eb</sup> in our transgenic mice (Fig. 3.1). However, due to the short length of the RGS2<sup>eb</sup> transgene and difficulty in immunoblotting (Chidiac <i>et al.</i>, 2014), a limitation of our study is our inability to fully demonstrate an eIF2B-RGS2<sup>eb</sup> complex. Although RGS2<sup>eb</sup> expression is associated with a decrease in cardiac hypertrophy and improved cardiac function, and our previous <i>in vitro</i> studies suggest that this is due to the inhibition of eIF2Bε, we cannot definitively attribute our <i>in vivo</i> observations to the direct inhibition of protein synthesis, and thus cannot exclude other off target effects as the mechanism behind our observed cardioprotection.

Although LV free wall thickness was significantly increased in both WT and RGS2<sup>eb</sup> TG mice following 4 weeks of TAC (Fig. 3.5C), HWT/BWT ratios indicated the development of significant TAC-associated cardiac hypertrophy in WT, but not RGS2<sup>eb</sup> TG mice (Fig. 3.5A). Furthermore, measurements of cardiomyocyte cross-sectional after area showed a corresponding increase in cell size for WT TAC but not RGS2<sup>eb</sup> TG mice (Fig. 3.5E). These data suggest that <i>de novo</i> protein synthesis in response to pressure overload, and thus hypertrophy, is inhibited in RGS2<sup>eb</sup> TG mice.

Our previous work showed that the adenoviral expression of RGS2<sup>eb</sup> in neonatal ventricular myocytes was able to attenuate the expression of genetic markers of hypertrophy to a comparable extent as full-length RGS2 (Chidiac <i>et al.</i>, 2014). Thus, we examined whether RGS2<sup>eb</sup> could also attenuate <i>in vivo</i> increases in the “fetal gene program”, a group of molecular markers associated with the hypertrophy phenotype, including β-MHC, ANP, and BNP (Molkentin and Dorn, 2001; Frey and Olson, 2003; Petrich and Wang, 2004). Four weeks of aortic constriction induced an increase in the levels of β-MHC and ANP in WT mice (Fig. 3.7). These increases were completely abrogated in RGS2<sup>eb</sup> TG animals. Reactivation of fetal gene expression during cardiac hypertrophy is initially an adaptive process which increases the excitability, contractility, and plasticity of cardiac myocytes in response to pathological stress (Kuwahara, Nishikimi and Nakao, 2012). However, sustained fetal gene expression plays a
causative role in maladaptive cardiac remodelling, leading to cardiac dysfunction and often the pathogenesis of heart failure (Hannenhalli et al., 2006). In addition, our assessment of cardiac function obtained from pressure-volume loop analyses also suggests that RGS2\textsuperscript{eb} TG mice were able to maintain significantly improved cardiac function after TAC compared to WT mice, as evidenced by both systolic and diastolic indices (Fig. 3.4). Our data suggest that the expression of RGS2\textsuperscript{eb} appears to not only inhibit cardiac hypertrophy, but also improves cardiac function without activation of fetal cardiac genes after 4 weeks of TAC, although the effect on fetal gene expression may more likely be due to an indirect effect of the inhibition of cardiac hypertrophy and apparent improvement in cardiac function, rather than a direct mechanistic connection between RGS2\textsuperscript{eb} and the re-expression of fetal genes. Further studies, such as prolonged exposure to TAC, may clarify the extent to which these effects are sustained.

Following transverse aortic constriction, RGS2 knockout mice have been found to develop severe cardiac hypertrophy and heart failure, as well as increased mortality compared to corresponding controls (Takimoto et al., 2009). Although this clearly indicates that the presence of RGS2 is cardioprotective in vivo, many details of this beneficial effect remain to be elucidated (Chidiac et al., 2014). In cultured ventricular myocytes, RGS2 is acutely and selectively upregulated in response to activators of G\textsubscript{q} and G\textsubscript{s} signalling (Hao et al., 2006; Zou et al., 2006; Nunn et al., 2010). However, RGS2 has been found to be ultimately downregulated in vivo following experimentally-induced hypertrophy and heart failure (Zhang et al., 2006). Furthermore, cardiac-specific overexpression of full-length RGS2 does not result in demonstrable cardioprotective effects in mice subjected to TAC (Park-Windhol et al., 2012). Conversely, the overexpression of either RGS4, RGS5, or RGS10 has been shown to reduce cardiac hypertrophy (Rogers et al., 1999, 2001; Li et al., 2010; Miao et al., 2016), although it may be noted that unlike RGS2, these proteins tend to localize to the atria rather than the ventricles (Doupnik, Xu and Shinaman, 2001; Mittmann et al., 2002; Larminie et al., 2004; Gu et al., 2009; Miao et al., 2016). It is possible that the optimal level of RGS2 is decreased under conditions of hypertrophic stress, however a complete lack of RGS2 is clearly detrimental under pressure overload (Takimoto et al., 2009).
The lack of an observable protective effect of full-length RGS2 overexpression in the myocardium (Park-Windhol et al., 2012) stands in contrast to the present findings that expression of its isolated eIF2B-interacting domain (RGS2eb) can protect against pathological cardiac hypertrophy and dysfunction in vivo. We have previously shown that the ability of RGS2eb to inhibit drug-induced cell hypertrophy is independent of the G-protein inhibitory effects of full-length RGS2 (Chidiac et al., 2014). Along with our current findings, it follows that the RGS2eb region may be an important contributor to the reported beneficial cardiac effects of RGS2. Since our previous studies suggest that the RGS2eb region specifically binds to eIF2B and thereby inhibits protein synthesis (Nguyen et al., 2009), it is possible that some function of full length RGS2 may produce detrimental effects in the context of pathological hypertrophy, consistent with its observed downregulation in the latter condition (Zhang et al., 2006). Negative RGS2 effects could conceivably arise from its inhibition of beneficial G protein-mediated signals (e.g. the protective effects of adenosine) or alternatively via its effects on non-G protein targets such as tubulin or TRP channels (Heo et al., 2006; Schoeber et al., 2006). To examine the importance of the RGS2eb region per se, future studies would be in order, with expression of this truncated form targeted to the myocardium of full-body or cardiac-specific RGS2 knockout mice.

3.5 Conclusions

The present results suggest that expression of the eIF2B-interacting domain of RGS2, RGS2eb, can protect against pathological cardiac hypertrophy and dysfunction in vivo, and that this may be via the direct inhibition of protein synthesis. We have previously shown the ability of RGS2eb to inhibit drug-induced cardiomyocyte hypertrophy; along with our current findings, this suggests that the RGS2eb region is an important contributor to the reported beneficial cardiac effects of RGS2.

Currently, the majority of drugs for cardiovascular diseases target GPCRs. Although these medications can offer significant benefits to patients, heart failure continues to be a global leading cause of morbidity and mortality. A better understanding of the various components
that are part of the GPCR signaling pathways may lead to improved therapeutics, and interest in RGS2 and other RGS proteins as possible drug targets is on the rise (Le and Coffman, 2003; Sjögren and Neubig, 2010; Sjögren, Blazer and Neubig, 2010; Sjogren et al., 2012; Turner et al., 2012; Go et al., 2013; Sjögren et al., 2016). In summary, this study has demonstrated that RGS2 is important in regulating in vivo cardiac hypertrophy and physiology, and suggests that this region of full-length RGS2 may be a key component of its reported cardioprotective effects.
References


Razeghi, P., Young, M. E., Alcorn, J. L., Moravec, C. S., Frazier, O. H. and Taegtmeyer, H.


EFFECTS OF TRANSVERSE AORTIC CONSTRICTION AND DRUG INDUCED EXPERIMENTAL PRESSURE OVERLOAD HYPERTROPHY ON $Rgs2^{-/-}$ MICE WITH CARDIOMYOCYTE SPECIFIC OVEREXPRESSION OF RGS2$^{eb}$
4.1 Introduction

Regulator of G protein signalling (RGS) proteins function as GTPase activating proteins (GAPs) to limit the intensity and duration of GPCR-mediated signalling. RGS2 is a member of the R4 subfamily of RGS proteins, and selectively inhibits $G_q$-mediated signalling via its GAP activity and also via a GAP-independent mechanism (Abramow-Newerly et al., 2006). RGS2 can also inhibit $G_s$ signalling through its direct binding to some adenylyl cyclase isoforms and possibly also via its binding to $G_{as}$ (Abramow-Newerly et al., 2006). Additionally, effects on $G_{i/o}$ have also been reported (Chakir et al., 2011) notwithstanding the relatively low affinity of RGS2 for $G_{ai/o}$ proteins (Heximer et al., 1999). Since $G_q$- and $G_s$-mediated signalling play well established roles in the development of cardiovascular disease, the ability of RGS2 to selectively inhibit these pathways makes it an important target of study (Heximer et al., 1997, 1999; Roy, Baragli, et al., 2006).

Pathological cardiac hypertrophy, which often results from chronic hypertension, is a leading risk factor for various cardiovascular conditions (Levy et al., 1990; Cipriano et al., 2001); aside from age, left ventricular hypertrophy is the most reliable predictor of poor cardiovascular outcomes in hypertensive patients, and is an independent risk factor for sudden death, stroke, coronary heart disease, and heart failure (Gardin et al., 2001; Verdecchia et al., 2001; Gradman and Alfayoumi, 2006). Thus, identifying regulators of pathological hypertrophy is of great interest and importance. Studies in vivo and in cardiomyocyte models of hypertrophy have shown that increased cell growth caused by prolonged $G_q$-coupled receptor stimulation, such as that induced by phenylephrine, can be inhibited by RGS2 overexpression (Roy, Nunn, et al., 2006; Zou et al., 2006), with a similar effect seen against $G_s$-mediated cardiomyocyte hypertrophy induced by isoproterenol (Nunn et al., 2010). The importance of RGS2 in the regulation of hypertrophy has been further demonstrated in knockout animal studies. In $Rgs2^{-/-}$ mice, experimentally induced pressure overload via transverse aortic constriction causes severe hypertrophy, heart failure, and death (Takimoto et al., 2009). In addition, chronic angiotensin II infusion has been shown to significantly increase blood pressures in $Rgs2^{-/-}$ mice compared to WT controls (Hercule et al., 2007).
While the G protein inhibitory effects of RGS2 are well established, RGS2 can also bind to and regulate other targets, including TRPV6 calcium channels and tubulin (Wang et al., 2005; Heo et al., 2006; Schoeber et al., 2006). We have previously shown that RGS2 can also bind to the ε subunit of eukaryotic initiation factor 2B (eIF2B), a component of the rate-limiting step of the initiation of mRNA translation (Nguyen et al., 2009). This property of RGS2 has been mapped to a 37 amino acid domain (residues 79-115) termed RGS2eb that is homologous to a region in the β subunit of eIF2 (Nguyen et al., 2009), and we have also demonstrated that RGS2eb expression in cultured neonatal cardiomyocytes is able to inhibit both protein synthesis and agonist induced hypertrophy at levels comparable to full-length RGS2 (Chidiac et al., 2014).

Recently, we reported that following four weeks of transverse aortic constriction (TAC), mice with cardiomyocyte specific overexpression of RGS2eb (herein referred to as Rgs2+/ε(eb) mice) were protected from pressure overload-induced cardiac hypertrophy, and maintained heart function at significantly improved levels compared to WT counterparts (see Chapter 3) (Lee et al., 2017). Reactivation of the fetal gene response was suppressed in Rgs2+/+/(eb) mice following TAC, and cardiomyocyte size was decreased in Rgs2+/+/(eb) mice compared to WT TAC controls. These findings suggest that in addition to its G-protein inhibitory actions, the RGS2eb region may contribute to the cardioprotective effects of full-length RGS2 in vivo via the inhibition of de novo protein synthesis.

Since full-length RGS2 was still being endogenously expressed in the Rgs2+/+/(eb) mice from our previous studies (Lee et al., 2017), the use of Rgs2−/− mice would provide further insights into the cardioprotective role of the RGS2eb region. Based on our previous findings, we hypothesized that the cardiomyocyte-specific overexpression of RGS2eb in Rgs2−/− mice could decrease the severity of cardiac hypertrophy and heart failure resulting from experimentally induced pressure overload, thereby compensating for the absence of full-length RGS2. Here we report the generation of novel transgenic Rgs2−/− mice with cardiomyocyte specific overexpression of RGS2eb (Rgs2−/−(eb)). Unexpectedly, we observed that the mortality rate for Rgs2−/− animals significantly exceeded that of WT controls following 28 days of transverse aortic constriction (TAC), suggesting an impaired ability to recover from cardiovascular insult.
Due to the unexpectedly high mortality rate of $\text{Rgs2}^{-/-}$ mice, the TAC-induced model of pressure overload was determined to be unsuitable for the evaluation of cardiac hypertrophy, and discontinued before the addition of $\text{Rgs2}^{+/-(eb)}$ mice to the study. The effects of drug-induced models of hypertension on $\text{Rgs2}^{-/-}$ mice were also evaluated. Chronic infusion of angiotensin II via osmotic pumps for 28 days (1.44 mg/kg/day) significantly increased blood pressures of experimental animals ($\text{Rgs2}^{+/-(eb)}$, $\text{Rgs2}^{-/-}$, and $\text{Rgs2}^{-/-}(eb)$), except for WT mice. Although cardiac function was impaired in $\text{Rgs2}^{-/-}$ animals compared to basal levels, cardiac hypertrophy was not observed in any experimental groups compared to saline controls. Further studies with angiotensin II infusion at higher concentrations (3.0 mg/kg/day for 14 days) in WT and $\text{Rgs2}^{-/-}$ mice also failed to induce clearly detectable cardiac hypertrophy. Additional pilot studies were subsequently performed using twice daily injections of isoproterenol (ISO) for 14 days, with preliminary results suggesting that a full study with prolonged injections beyond 14 days may result in cardiac hypertrophy. Overall, owing in large part to the general lack of efficacy and/or safety of established methods with respect to the induction of cardiac hypertrophy in our experimental animals, our results do not support our original hypothesis that $\text{RGS2}^{eb}$ expression in an $\text{RGS2}$ null background would provide a “rescue” effect. In addition, the present findings provide little if any support for the notion that mice lacking $\text{RGS2}$ exhibit greater levels of experimentally-induced cardiac hypertrophy.

4.2 Methods

4.2.1 Generation of $\text{Rgs2}^{-/-}$ mice with cardiac specific overexpression of $\text{RGS2}^{eb}$

$\text{Rgs2}^{+/-(eb)}$ were crossed with $\text{Rgs2}^{-/-}$ mice to produce heterozygous $\text{Rgs2}^{+/+}$ animals with or without the expression of $\text{RGS2}^{eb}$ ($\text{Rgs2}^{+/-(eb)}$ or $\text{Rgs2}^{+/+}$, respectively). $\text{Rgs2}^{+/+}(eb)$ mice were then crossed with $\text{Rgs2}^{-/-}$ mice to obtain $\text{RGS2}$ knockout animals expressing $\text{RGS2}^{eb}$ ($\text{Rgs2}^{-/-}(eb)$). Subsequent crosses of $\text{Rgs2}^{-/-}(eb)$ with $\text{Rgs2}^{-/-}$ animals enabled the establishment of a mouse colony which produced $\text{Rgs2}^{-/-}(eb)$ mice and $\text{Rgs2}^{-/-}$ littermates (Fig. 4.1).
Colonies for Rgs2−/− and Rgs2+/+(eb) mice are well established in our laboratory (Roy, Nunn, et al., 2006; Lee et al., 2017). Briefly, during our initial generation of Rgs2+/+(eb) mice, the RGS2eb gene was targeted to the heart using the mouse α-MHC promoter (kindly provided by Jeffrey Robbins, Cincinnati Children’s Hospital Medical Center) (Subramaniam et al., 1991). Primers for the detection of transgenic Rgs2+/+(eb) mice and control primers for endogenous RGS2 expression have been previously described in Chapter 3 (Lee et al., 2017); the genotyping of Rgs2−/− mice has also been previously described (Oliveira-Dos-Santos et al., 2000). Three to five month old male mice were used for experimental studies; all mouse lines are in C57Bl/6 backgrounds. Animals were maintained in accordance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. These studies were approved by the Animal Care Committee at the University of Western Ontario, and complied with the guidelines of the Canadian Council on Animal Care.

4.2.2 Transverse aortic constriction (TAC)

TAC was used to induce pressure overload on the hearts of 12 week old male C57BL/6 wild-type (i.e., Rgs2+/+) and Rgs2−/− mice, and as described in Chapter 3 (Lee et al., 2017). Briefly, two knots were tied against a 25-gauge blunt needle placed parallel to the transverse aorta, which was immediately removed after the second tied knot followed by closure of the chest. Control WT and Rgs2−/− mice were subjected to sham operations without aortic constriction.

4.2.3 Drug treatments

28 day angiotensin (Ang) II infusion: Ang II (A9525, Sigma-Aldrich, Canada) was dissolved in sterile saline and added to osmotic pumps (Model 1004, Alzet, New York, NY, USA) as per manufacturer’s protocols (Alzet, 2017a); control pumps were filled with saline. The pump filling concentration of Ang II required to deliver the drug at a dose of 1.44 mg/kg/d was calculated using the mean pumping rates for each lot of osmotic pumps and the Alzet drug concentration calculator (Alzet, 2017b). Pumps were implanted subcutaneously under isoflurane anaesthesia. Echocardiography and blood pressure measurements were performed for pre-implantation (basal), 14 day, and 28 day time points. Experimental animals included
Rgs2−/− and Rgs2−/−(eb) animals, as well as control WT and Rgs2+/+(eb) mice. Animals were sacrificed after 28 days for tissue collection.

14 day angiotensin II infusion: Ang II (12-1-34, American Peptide Company, CA, USA) was dissolved in sterile saline and added to osmotic pumps (Model 1002, Alzet); control pumps were filled with saline. The pump filling concentration of Ang II required to deliver the drug at a dose of 3.0 mg/kg/d was as described above. Echocardiography was performed on day 14, followed by animal sacrifice and tissue collection. Only Rgs2−/− and WT mice were used for this study.

Isoproterenol (ISO) injections: ISO (I6504, Sigma-Aldrich) was dissolved in sterile saline and injected subcutaneously at a dose of 15 mg/kg or 50 mg/kg twice daily (at 0900 h and 2100 h) for 12 days or 14 days, respectively; control mice were injected with sterile saline. Experimental animals included Rgs2−/− and Rgs2−/−(eb) animals, as well as control WT and Rgs2+/+(eb) mice. Mice were weighed daily and sacrificed on day 12 or day 14 for tissue collection.

4.2.4 Assessment of blood pressure

Non-invasive tail-cuff blood pressures and heart rates were measured using the CODA tail-cuff blood pressure system (Kent Scientific, USA) as previously described (Tutunea-Fatan et al., 2015). For angiotensin II osmotic pump studies, heart rates and tail-cuff blood pressures were recorded in conscious mice for basal, 14 day, and 28 day time points.

4.2.5 Assessment of cardiac function

M-mode (motion mode) echocardiography was performed after Ang II or saline treatments as previously described (Liu et al., 2014; Roy et al., 2016). Briefly, cardiac function was measured using the Vevo 2100 imaging system (VisualSonics, Canada). Using a dynamically focused 40MHz probe, 2-dimensional heart images were obtained in short-axis view for the left ventricle (LV).

M-mode recordings were assessed for the determination of various cardiovascular parameters using the Cardiac Measurement Package (PSLAX protocol) and averaged from 3 cycles
Anterior and posterior wall thickness, as well as LV internal diameter were directly measured from M-mode recordings for both systolic and diastolic phases. Ejection fraction, fractional shortening, LV volume, and corrected LV mass were derived from established formulas calculated by the VisualSonics analysis report function (VisualSonics, 2008). Detailed measurement and calculation definitions from VisualSonics are described in Supplemental Table C1.

### 4.2.6 Tissue collection

Upon completion of studies, anaesthetized mice were euthanized via a 10% KCl injection into the left jugular vein to ensure cardiac arrest in the diastolic state. Hearts were excised, weighed after removal of the atria, and stored at -80°C for subsequent RNA isolation. For 50 mg/kg ISO experiments, lungs were also excised and rinsed in saline, then weighed. Tibias from each animal were excised, cleaned, and measured for heart weight/tibia length calculations.

### 4.2.7 Quantitative reverse transcriptase qPCR

Total RNA was isolated from LV heart tissue using the TriZol (Invitrogen) extraction method for 1.44 mg/kg/day Ang II experiments. Reverse transcription reaction was performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Invitrogen, Canada). SensiFAST SYBR No-ROX Kit mastermix (FroggaBio, Canada) was used for real-time thermal cycling. Primers for β-MHC, ANP, and BNP were previously described in Chapter 3 (Lee et al., 2017), and were designed using the OligoPerfect Designer (ThermoFisher, Canada), OligoCalc (Kibbe, 2007) and Primer-BLAST (NCBI) programs. Samples were amplified for 35 cycles using a CFX384 real-time thermal cycler (Bio-Rad, Canada). The mRNA quantity for each gene of interest was determined using standard curve analysis and normalized to 28S ribosomal expression.

### 4.2.8 Statistical analysis

All data were analyzed using GraphPad Prism 6.01 (GraphPad). Statistical analyses were performed using two-way ANOVA followed by Bonferroni post-tests, Log rank test (for
survival curves), or Student’s t-test and presented as mean ± SEM. Differences were considered significant at P<0.05.

4.3 Results

4.3.1 Generation of transgenic mice with cardiomyocyte-specific overexpression of RGS2eb

We previously demonstrated that the *vivo* expression of RGS2eb inhibited pressure overload induced cardiac hypertrophy and improved cardiac function (Lee *et al.*, 2017). Since endogenous RGS2 was still present in our previous studies, we used well established Rgs2−/− and Rgs2+/+(eb) mouse lines in our laboratory to develop a novel strain of Rgs2−/− mice with targeted myocardial overexpression of RGS2eb (Rgs2−/−(eb)) (Fig. 4.2A). As previously mentioned in Chapter 3, genotyping with primers for a portion of the hGH polyA region, which was only present in the transgenic sequence of Rgs2+/+(eb) mice, was used to identify mice expressing RGS2eb. Primers for endogenous RGS2 and a region of the RGS2 knockout construct (Oliveira-Dos-Santos *et al.*, 2000), were used to identify wildtype and Rgs2−/− animals, respectively. Together, these results indicate the successful generation of Rgs2−/− mice with cardiomyocyte specific overexpression of RGS2eb.

4.3.2 Increased mortality in Rgs2−/− mice following pressure overload

Transverse aortic constriction (TAC) was used to induce experimental pressure overload on wildtype and Rgs2−/− mice. Since Rgs2−/− mice have been previously reported to be highly sensitive to pressure overload induced hypertrophy and heart failure (Takimoto *et al.*, 2009), initial TAC studies were performed with Rgs2−/− animals to establish whether the previous surgery conditions used in Chapter 3 were suitable. Survival rates for Rgs2−/− mice subjected to TAC surgery were found to be significantly lower than sham controls, with 70% of Rgs2−/− mice dying within two days of TAC surgery (Fig. 4.2A); a similar effect was not observed for WT TAC mice (Fig. 4.2B). Due to the low survival rate of Rgs2−/− mice, we concluded that TAC was too severe of a procedure for our goal of producing a hypertrophy model. Due to
these observations, we did not continue this study with our remaining experimental groups (i.e. with Rgs2\(^{+/+}\) (eb) and Rgs2\(^{-/-}\) mice).

### 4.3.3 28 day angiotensin II treatment at 1.44 mg/kg/day

As an alternative to TAC, we investigated the effects of drug-induced hypertension in Rgs2\(^{-/-}\) (eb) and Rgs2\(^{-/-}\) mice. Chronic Ang II infusion has been previously shown to reproducibly increase blood pressure and induce the development of cardiac hypertrophy in C57Bl/6 mice (Francois et al., 2004; Crowley et al., 2006). Tail cuff systolic and diastolic blood pressure measurements as well as HR were taken one to five days prior to pump implantation (basal) and at 14 days and 28 days following infusion of saline or Ang II at a dose of 1.44 mg/kg/day in WT, Rgs2\(^{+/+}\) (eb), Rgs2\(^{-/-}\), and Rgs2\(^{-/-}\) (eb) mice. In Ang II treated Rgs2\(^{+/+}\) (eb) mice, significant increases in systolic BP were observable by day 14 and remained elevated through day 28 compared to basal levels (Fig. 4.3A) Similar significant systolic BP changes were observed at day 28 in Rgs2\(^{-/-}\) and Rgs2\(^{-/-}\) (eb) mice (Fig. 4.3A). However, WT systolic BP Ang II treatment at 28 days was not significantly increased compared to basal (P=0.062, Fig. 4.3A). Significant elevations in diastolic BP were observed at 28 days in Rgs2\(^{-/-}\) and Rgs2\(^{-/-}\) (eb) mice (Fig. 4.3B). No changes in systolic or diastolic BPs occurred in saline controls (Fig. 4.3C and D), and heart rates were comparable between all genotypes and treatment groups (Table 4.2).

Although hypertension was evident in WT, Rgs2\(^{+/+}\) (eb), Rgs2\(^{-/-}\), and Rgs2\(^{-/-}\) (eb) mice following chronic Ang II treatment, cardiac hypertrophy was not observed in any strains or treatment groups following the 28 day time point, as measured by either heart weight/tibia length (Fig. 4.4A and C) or echocardiography for LV mass (Fig. 4.4C and D). In addition, echocardiography measurements for LV diastolic and systolic internal diameters (LVID) in saline and Ang II treated groups did not provide evidence for chamber dilation or hypertrophy (Figure 4.5). Echocardiography measurements for posterior and anterior LV wall thickness during diastole and systole provided further evidence for a lack of cardiac hypertrophy (Figure 4.6 and 4.7); LV volume was also comparable to basal levels (Figure 4.8). Overall, these results suggest that experimental hypertension induced by Ang II infusion was insufficient for the development of cardiac hypertrophy in any of the groups tested.
4.3.4 Decreased cardiac function in \textit{Rgs2}^{-/-} mice following 28 days of angiotensin II treatment

In addition to hypertrophy parameters, cardiac function was also assessed in WT, \textit{Rgs2}^{+/+}(eb), \textit{Rgs2}^{-/-}, and \textit{Rgs2}^{-/-}(eb) mice following 28 days of saline of Ang II infusion. Ejection fraction (EF) and fractional shortening (FS) in \textit{Rgs2}^{-/-} mice were significantly decreased compared to \textit{Rgs2}^{-/-} basal values (Fig. 4.9A and C). No significant decreases in EF or FS were observed in WT, \textit{Rgs2}^{+/+}(eb), or \textit{Rgs2}^{-/-}(eb) following Ang II or saline treatment (Fig. 4.9).

4.3.5 Expression of fetal genes in LV tissue of 28 day 1.44 mg/kg/day chronic angiotensin II treated mice

Induction of “fetal” cardiac genes is often a common feature in animal models of pathological hypertrophy and heart failure (Parker, Packer and Schneider, 1990; Razeghi \textit{et al.}, 2001). RNA was isolated from left ventricular tissue after 28 days of Ang II or saline, and qPCR was performed to determine expression levels of β-myosin heavy chain (β-MHC), atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) in WT and \textit{Rgs2}^{-/-} mice. Surprisingly, WT mice treated with Ang II were found to express significantly lower levels of β-MHC compared to Ang II \textit{Rgs2}^{-/-} animals and saline controls (Fig. 4.10 A). ANP was significantly increased in \textit{Rgs2}^{-/-} compared to WT Ang II mice (Fig. 4.10B), while BNP was significantly decreased in \textit{Rgs2}^{-/-} compared to WT saline mice (Fig. 4.10 C).
Figure 4.1. Breeding schematic for generation of *Rgs2*<sup>-/-</sup>(eb) mice

*Rgs2*<sup>-/-</sup> and *Rgs2*<sup>+/-(eb)</sup> mouse lines were crossed to obtain heterozygous mice with transgene expression (*Rgs2*<sup>+-/(eb)</sup>). Subsequent crosses with *Rgs2*<sup>-/-</sup> mice produced a colony of *Rgs2*<sup>-/-</sup> and *Rgs2*<sup>-/-/(eb)</sup> littermates.
Figure 4.2. Survival and cardiac hypertrophy following 28 days TAC in WT and Rgs2/- mice

(A) Significantly lower survival was observed for Rgs2/- TAC mice compared to sham controls, which was not seen in (B) WT TAC animals. WT survival data is based on sham and TAC surgeries completed for Chapter 3 studies. Data represent means ± SEM, n = 5-22 per group. *P<0.05 and ***P<0.001 using Log rank test for survival curves.
TG and KOT designations indicate Rgs2^{+/+(eb)} and Rgs2^{-/-(eb)} mice, respectively. (A) Systolic and (B) diastolic blood pressures (BP) were significantly altered at multiple time points in Ang II treated mice. (C) Systolic and (D) diastolic BPs for saline treated mice remained stable throughout the 28 days. Data represent means ± SEM, n=8-18 per genotype group for Ang II, and n=2 per genotype group for saline treated. *P<0.05 and ***P<0.001 using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.

Figure 4.3. Tail-cuff blood pressure measurements for basal, 14 day, and 28 days during saline of Ang II 1.44 mg/kg/day.
Table 4.1. Heart rates for Ang II and saline treated WT, Rgs2<sup>+/+(eb)</sup>, Rgs2<sup>−/−</sup>, and Rgs2<sup>−/−(eb)</sup> mice (beats per minute)

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
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<td>WT</td>
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<td>n</td>
<td>2</td>
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<tr>
<td>Basal</td>
<td>673</td>
<td>670</td>
<td>639</td>
<td>636</td>
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<tr>
<td>14 day</td>
<td>687</td>
<td>645</td>
<td>641</td>
<td>617</td>
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<tr>
<td>28 day</td>
<td>632</td>
<td>672</td>
<td>671</td>
<td>607</td>
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<td>Ang II</td>
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<td>5-16</td>
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<tr>
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<td>690±14</td>
<td>670±12</td>
<td>639±19</td>
<td>636±47</td>
<td></td>
</tr>
<tr>
<td>14 day</td>
<td>704±25</td>
<td>660±31</td>
<td>651±22</td>
<td>607±27</td>
<td></td>
</tr>
<tr>
<td>28 day</td>
<td>681±42</td>
<td>707±42</td>
<td>653±43</td>
<td>604±23</td>
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TG and KOT columns indicate Rgs2<sup>+/+(eb)</sup> and Rgs2<sup>−/−(eb)</sup> mice, respectively.
Heart weight/tibia length was measured for WT, Rgs2\(^{+/+}\) (TG), Rgs2\(^{-/-}\) (KO) and Rgs2\(^{-/-}\)(eb) (KOT) mice in Ang II and saline treated after 28 days. LV mass was also measured from echocardiography images at basal, 14 days, and 28 days of treatment. Data represent means ± SEM, n=3 (saline) or 8 (Ang II-treated) per genotype group. Statistical analysis was performed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
(A)(B) LVID;d (C)(D) LVID;s were comparable between both Ang II and saline treatment groups, as well as between genotypes for WT, Rgs2\(^{+/+}(eb)\) (TG), Rgs2\(^{-/-}\) (KO) and Rgs2\(^{-/-}(eb)\) (KOT) mice. LVID was measured from echocardiography images at basal, 14 days, and 28 days of treatment. Data represent means ± SEM, n=3 (saline) or 8 (Ang II treated) per genotype group. Statistical analysis was performed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
Figure 4.6. Anterior wall (AW) thickness, diastole and systole, 1.44 mg/kg/day Ang II or saline treatment

(A) (B) AW;d and (C) (D) AW;s were comparable between both Ang II and saline treatment groups, as well as between genotypes for WT, Rgs2<sup>+/+<sub>(eb)</sub></sup> (TG), Rgs2<sup>−/−</sup> (KO) and Rgs2<sup>−/−(eb)</sup> (KOT) mice. AW was measured from echocardiography images at basal, 14 days, and 28 days of treatment. Data represent means ± SEM, n=3 (saline) or 8 (Ang II treated) per genotype group. Statistical analysis was performed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
Figure 4.7. Posterior wall thickness (PW), diastole and systole, 1.44 mg/kg/day Ang II or saline treatment

(A) (B) PW;d and (C) (D) PW;s were comparable between both Ang II and saline treatment groups, as well as between genotypes for WT, Rgs2<sup>+/+(eb)</sup> (TG), Rgs2<sup>-/-</sup> (KO) and Rgs2<sup>-/-</sup>(eb) (KOT) mice. PW was measured from echocardiography images at basal, 14 days, and 28 days of treatment. Data represent means ± SEM, n=3 (saline) or 8 (Ang II treated) per genotype group. Statistical analysis was performed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
(A) (B) LV;d and (C) (D) LV;s were comparable between both Ang II and saline treatment groups, as well as between genotypes for WT, Rgs2<sup>+/+(eb)</sup> (TG), Rgs2<sup>−/−</sup> (KO) and Rgs2<sup>−/−(eb)</sup> (KOT) mice. LV was measured from echocardiography images at basal, 14 days, and 28 days of treatment. Data represent means ± SEM, n=3 (saline) or 8 (Ang II treated) per genotype group. Statistical analysis was performed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.

Figure 4.8. LV vol, diastole and systole, 1.44 mg/kg/day Ang II or saline treatment
Figure 4.9. Ejection fraction and fractional shortening, 1.44 mg/kg/day Ang II or saline treatment

(A) EF in Rgs2−/− (KO) was significantly reduced after 28 days of ang compared to basal Rgs2−/− (KO) levels. Changes to EF compared to basal were not observed for WT, (B) Rgs2+/+ (TG) or Rgs2−/− (KOT) mice. Similarly, (C) FS was significantly reduced in Rgs2−/− (KO) was significantly reduced after 28 days of ang compared to basal, with no changes observed in WT, (D) Rgs2+/+ (TG) or Rgs2−/− (KOT) mice. EF and FS were calculated from LV vol and LVID parameters (see Suppl. Table C1 for equations) at basal, 14 days, and 28 days of treatment. Data represent means ± SEM, n=3 (saline) or 8 (Ang II treated) per genotype group. Statistical analysis was performed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
Figure 4.10. Markers of cardiac hypertrophy

(A) After 28 days of Ang II or saline, WT mice treated with Ang II were found to express significantly lower levels of β-MHC compared to Ang II Rgs2−/− animals and saline controls. (B) ANP was significantly increased in Rgs2−/− compared to WT Ang II mice, while (C) BNP was significantly decreased in Rgs2−/− compared to WT saline mice (Fig. 4.10 C). Data represent means ± SEM, n=6 per group. *P<0.05 and **P<0.01 using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
4.3.6  14 day angiotensin II treatment at 3.0 mg/kg/day

Since Ang II treatment at 1.44 mg/kg/day was sufficient to increase blood pressure, but did not appear to induce cardiac hypertrophy, 3.0 mg/kg/day of Ang II was tested in WT and Rgs2\(^{-/-}\) mice for 14 days to determine whether an increase in drug dosage could produce a hypertrophic response. Significant cardiac hypertrophy did not occur in either WT or Rgs2\(^{-/-}\) mice compared to saline controls (Fig. 4.11A). Furthermore, echocardiography for systolic and diastolic diameters (Fig. 4.11 B and C), as well as EF and FS (Fig. 4.11D and E), were similar between all treatment groups and strains.

4.3.7  Effect of twice daily isoproterenol injections

Due to a lack of inducible cardiac hypertrophy in our Ang II models, we tested whether a different drug and mode of delivery could improve our attempts to characterize the Rgs2\(^{+/- (eb)}\) mouse. Isoproterenol was injected subcutaneously at a dose of 15 mg/kg for 12 days. To determine feasibility, initial tests were performed only in WT mice. Following ISO injection, WT heart rates (under anaesthesia) rapidly increased from approximately 400 bpm to 700 bpm thirty seconds after injection and plateaued at that level (Fig. 4.12A), indicating that a pharmacologically relevant dose was used. HR measurements 8 hours and 24 hours after ISO delivery showed a gradual return to baseline, suggesting that twice daily doses of ISO would be appropriate to maintain animal HRs at elevated levels (Fig. 4.12A). Following twelve days of ISO or saline injections, statistically significant levels of hypertrophy were not observed (data not shown). Therefore, ISO dosage was subsequently increased to 50 mg/kg twice daily for 14 days to WT, Rgs2\(^{+/- (eb)}\), Rgs2\(^{-/-}\), and Rgs2\(^{+/- (eb)}\) mice; body weights were recorded daily and animals were tracked for survival (Suppl. Fig. C1). Wet lung weight/tibia length was similar between all strains and conditions, and did not suggest the development of congestion (Fig. 4.13A). Similar to the twelve day ISO studies, the development of hypertrophy in Rgs2\(^{-/-}\) mice compared to saline controls was not statistically significant (P=0.08) (Fig. 4.13B).
4.4 Discussion

Maladaptive cardiac hypertrophy is a common condition that often develops in settings of hypertension, and is a major risk factor for heart failure and sudden death. Since the heart is thought to be an essentially post mitotic organ, cardiomyocyte growth requires de novo protein synthesis, which is controlled primarily at the initiation of mRNA translation stage (Sugden et al., 2009). We have previously shown that the in vitro expression of RGS2\textsuperscript{eb}, a region of full-length RGS2, can block protein synthesis and cultured cardiomyocyte growth by inhibiting eIF2B, a key component of the rate-limiting step of protein synthesis initiation (Nguyen et al., 2009; Chidiac et al., 2014). Furthermore, we recently developed a transgenic mouse line with cardiomyocyte specific overexpression of RGS2\textsuperscript{eb} (i.e. Rgs2\textsuperscript{+/-+eb} mice). Using a pressure overload model, we demonstrated that expression of RGS2\textsuperscript{eb} inhibited TAC-induced increases in cardiac hypertrophy and the expression of hypertrophy-related gene markers, as well as associated functional loss (Lee et al., 2017). These results likely reflected the ability of RGS2\textsuperscript{eb} to inhibit protein synthesis in vivo, and the consequent suppression of the hypertrophic response and cardioprotective effects.

In the present studies, we attempted to extend our earlier work to study the potential protective effect of RGS2\textsuperscript{eb} expression targeted to the myocardium in whole body RGS2 knockout mice, but this effort was abandoned due to unacceptably high mortality of the TAC procedure in Rgs2\textsuperscript{-/-} animals. We also were unable to establish whether the hypothesized protective/rescue effect could be observed with drug-induced experimental cardiac hypertrophy due to generally poor efficacies of established hypertrophy-inducing drug treatments in our experimental animals. Finally, the majority of our results do not support the notion the Rgs2\textsuperscript{-/-} mice are prone to more severe experimental hypertrophy, in contrast to the previous findings of Takimoto and colleagues (Takimoto et al., 2009).

Our previous work demonstrated the anti-hypertrophic effects of RGS2\textsuperscript{eb} expression in a C57Bl/6 background where endogenous expression of full-length RGS2 (which contains the RGS2\textsuperscript{eb} region) was still present. The goal of the present study was to determine whether the apparent ability of RGS2\textsuperscript{eb} to confer cardioprotection could be extended in part to Rgs2\textsuperscript{-/-} mice, which have previously been reported to be highly sensitive to TAC induced pressure.
(A) Significant cardiac hypertrophy did not occur in WT or Rgs2−/− mice compared to saline controls. Echocardiography for (B) diastolic and (C) systolic parameters, as well as (D) ejection fraction (EF) and (E) fractional shortening (FS) were similar between all treatment groups and strains. Data represent means ± SEM, n=3-8 per group. Statistical analysis was performed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
Following ISO injection, WT heart rates (under anaesthesia) rapidly increased from approximately 400 bpm to 700 bpm thirty seconds after injection, and plateaued at that level. HR measurements 8 hours and 24 hours after ISO delivery showed a gradual return to baseline, suggesting that twice daily doses of ISO would be appropriate to maintain animal HRs at elevated levels. Data points represent individual animals.

Figure 4.12. ISO tests in WT mice, 15 mg/kg s.c. injections
(A) Wet lung weight/tibia length was similar between all strains and conditions, and did not suggest the development of congestion. (B) Although statistical significance was not attained, heart weight/tibia length ratios indicated the development of hypertrophy in $Rgs2^{-/-}$ (KO) and $Rgs2^{-/-}(eb)$ (KOT) mice. Data represent means ± SEM, n=3-8 per group. Statistical analysis was performed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.

Figure 4.13. Lung weight/tibia length and heart weight/tibia length in 14 day saline or 50 mg/kg ISO treated mice
overload (Takimoto et al., 2009). In order to develop a line of $Rgs2^{−/−}$ mice with cardiomyocyte specific overexpression of RGS2$^{eb}$ ($Rgs2^{-/+(eb)}$), we crossed $Rgs2^{+/+(eb)}$ and $Rgs2^{−/−}$ mice from well-established mouse lines in our laboratory to obtain intermediate animals with heterozygous RGS2 expression. Mice were genotyped to identify those with expression of the RGS2$^{eb}$ transgene, then crossed back with $Rgs2^{−/−}$ animals to obtain $Rgs2^{−/−(eb)}$ mice, which were subsequently crossed with $Rgs2^{−/−}$ animals to establish a novel $Rgs2^{−/−(eb)}/Rgs2^{−/−}$ mouse line (Figure 4.1).

High mortality rates of $Rgs2^{−/−}$ mice after TAC led to the discontinuation of our initial TAC studies. However, this was an interesting observation in itself (Fig. 4.2B). Previous studies using a more severe TAC model have also demonstrated lower survival rates in $Rgs2^{−/−}$ animals, albeit to a much smaller extent than our observations (60% survival versus 30%, respectively) (Takimoto et al., 2009). A possible explanation for these low survival rates is that $Rgs2^{−/−}$ mice have been shown to have increased susceptibility to atrial fibrillation (AF) via enhanced M3 muscarinic receptor activity, which is known to be involved in vagally induced atrial fibrillation (Tuomi, Chidiac and Jones, 2010; Jones, Tuomi and Chidiac, 2012). Studies have also shown that AF can be induced more frequently via electrical stimulation in wild-type TAC hearts compared to sham surgeries, and that this may be due to mast cell infiltration and fibrosis in the atrium following TAC (Liao et al., 2010). In addition, the use of a ketamine/xylazine anaesthetic mix, which depresses cardiac function (Kohn, 1997; Roth et al., 2002), may have also had an adverse effect. Thus, it is possible that after TAC surgery, a combination of the loss of RGS2 and additional physiological stressors induced atrial fibrillation and cardiac dysfunction, from which a majority of our $Rgs2^{−/−}$ animals did not recover.

Chronic Ang II infusion via osmotic pumps at both pressor and sub-pressor doses has been shown to induce hypertension and cardiac hypertrophy in various mouse lines including C57 Bl/6 mice (Griffin et al., 1991; Bendall et al., 2002; Schultz et al., 2002; Choi et al., 2016; Tsuruda et al., 2016). Although we observed hypertension in our experimental mice after 28 days of Ang II at 1.44 mg/kg/day (Fig. 4.3), we did not observe cardiac hypertrophy in any of our experimental or control mice (WT, $Rgs2^{+/+(eb)}$, $Rgs2^{−/−}$, and $Rgs2^{−/−(eb)}$). However, functional echocardiography data suggested significantly decreased ejection fraction and fractional
shortening in $Rgs2^{+/}$Ang II animals compared to basal $Rgs2^{+/}$ levels, which was not observed in the other experimental groups (Fig. 4.9). Levels of ANP were also significantly elevated in $Rgs2^{+/}$ mice compared to WT controls (Fig. 4.10B). These data suggested that although Ang II infusion was sufficient to increase blood pressure over 28 days, it was not, at least in our hands, able to reliably deliver a robust hypertension-induced model of cardiac hypertrophy. Therefore, we tested approximately double the concentration of Ang II (3.0 mg/kg/day) in WT and $Rgs2^{+/}$ mice for 14 days to assess whether a higher dose could induce cardiac hypertrophy and thus be used for additional studies; no statistically significant increases in heart weight were observed (Fig. 4.11). It is possible that our $Rgs2^{+/}$ (and potentially WT) mouse strains are, for currently unknown reasons, resistant to the development of cardiac hypertrophy, as the pressure overload and hypertrophy models used in our experiments are well established in the literature and in our own laboratories (Roy et al., 2016; Lee et al., 2017). Further studies, such as prolonged or higher doses of Ang II, may clarify the parameters required for a robust model of Ang II-induced cardiac hypertrophy.

Following a lack of significant hypertrophy in all our mouse strains after chronic Ang II treatment, we hypothesized that acute delivery of a hypertrophic agent could potentially result in the development of cardiac hypertrophy. Since our previous study used Ang II, which activates $G_q$-stimulated hypertrophic signalling pathways, we selected isoproterenol, a $G_s$ activating agonist ($\text{via } \beta$-adrenergic receptors), to ultimately administer in a pilot study at twice daily doses of 50 mg/kg for 14 days to WT, $Rgs2^{+/+}_{(eb)}$, $Rgs2^{+/-}$, and $Rgs2^{-/-}_{(eb)}$ mice. Initial heart weight/tibia length ratios following ISO treatment demonstrated no statistically significant differences between genotype or treatment groups, although ISO (Fig. 4.13B) has been previously shown to induce cardiac hypertrophy at concentrations ranging from 15 mg/kg to 100mg/kg (Zhang et al., 2005; Brooks and Conrad, 2009; Sjögren et al., 2016). Future studies with blood pressure monitoring as well as echocardiography analysis may help determine whether an isoproterenol model is the appropriate drug of choice for a hypertrophy model in our studies with $Rgs2^{-/-}_{(eb)}$ mice.
4.5 Conclusions

The present results are generally consistent with studies in the literature that suggest impaired cardiovascular function in $Rgs2^\gamma$ mice may be due to enhanced susceptibility to atrial fibrillation. However, our objective of determining whether the expression of RGS2$^{eb}$ in $Rgs2^\gamma$ mice ($Rgs2^{\gamma(eb)}$) could decrease previously reported deleterious outcomes following pressure overload was limited by our inability to achieve a robust cardiac hypertrophy model with appropriate experimental controls. Subsequent studies using well-established angiotensin and isoproterenol hypertrophy models also did not produce significant increases in heart weight in any of our mouse strains. It is possible that future studies with prolonged isoproterenol or angiotensin II treatment could result in conditions leading to a robust cardiac hypertrophy model in which the role of RGS2$^{eb}$ in $Rgs2^\gamma$ mice could be appropriately characterized.
4.6 References


5 LOSS OF REGULATOR OF G PROTEIN SIGNALLING 2 (RGS2) ALTERS THE EFFECTS OF ACUTE AND CHRONIC DIET RESTRICTION ON METABOLIC OUTCOMES IN C57 BL/6 MICE

5.1 Introduction

Regulator of G protein signalling (RGS) proteins are a family of GTPase-activating proteins (GAPs) that bind to G proteins and inactivate them by increasing the intrinsic rate of GTP hydrolysis and/or act as “effector antagonists”, thus limiting the intensity and duration of GPCR-initiated signalling (Popov et al., 1997; Ross and Wilkie, 2000; Abramow-Newerly et al., 2006). RGS2 is part of the B/R4 family of RGS proteins and is found ubiquitously throughout the body (Schoeber et al., 2006). RGS2 selectively inhibits G_{αq} and G_{αs}-mediated signalling (some effects on G_{αi/o} signalling have also been reported) (Heximer et al., 1999; Anger et al., 2007; Chakir et al., 2011), and can bind to and regulate other non-G protein targets including eIF2B, TRPV6 calcium channels and tubulin (Wang et al., 2005; Heo et al., 2006; Schoeber et al., 2006). RGS2 has also been shown to modulate adipocyte differentiation and proliferation (Imagawa, Tsuchiya and Nishihara, 1999; Nishizuka et al., 2001; Cheng et al., 2008), and may also regulate basal metabolism via the inhibition of thyroid hormone receptor-induced G_{αq} signalling (Eszlinger et al., 2004). Furthermore, human polymorphisms leading to increased RGS2 expression have been associated with metabolic syndrome and an increased risk for weight gain or obesity in hypertensive patients (Freson et al., 2007; Sartori et al., 2008). Conversely, Rgs2 deficiency in mice leads to impaired T cell activation, increased anxiety, reduced male aggression, and moderate hypertension under basal conditions (Oliveira-Dos-Santos et al., 2000; Heximer et al., 2003).

We have previously reported that Rgs2\(^{-/}\) mice exhibit a lean phenotype, and demonstrated that resistance to age-related weight gain was accompanied by reduced adipose tissue deposits and smaller adipocytes, lower levels of serum lipids, and increased insulin sensitivity; a subset of genes involved in adipocyte differentiation and proliferation were also reduced (Nunn et al., 2011). In addition, Rgs2\(^{-/}\) mice were found to be hyperphagic in comparison to C57 Bl/6 wildtype (WT) controls, and the two strains exhibited equivalent nutrient absorption (Nunn et al., 2011). A lean phenotype in the presence of hyperphagia and normal nutrient absorption would appear to suggest increased physical activity and/or metabolism, however we were not able in our previous study to detect any increase in resting VO\(_2\) in Rgs2\(^{-/}\) mice using a basic
open flow respirometry system where animals were held in a small chamber and had limited mobility (Nunn et al., 2011).

Although the lean $Rgs2^{-/-}$ habitus was maintained throughout the mouse lifespan on an *ad libitum* standard rodent diet, these animals nonetheless became obese and insulin-resistant under high-fat diet conditions (Nunn et al., 2011). This suggests that $Rgs2^{-/-}$ mice do not have a major impairment with respect to their ability to accumulate adipose tissue (evidence for a role of RGS2 in adipocyte differentiation notwithstanding). It follows that the observed lean phenotype may reflect underlying changes in energy utilization that limit substrate availability with respect to adipose formation under normal dietary conditions.

To better understand the role of RGS2 in metabolism and body weight regulation, Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH, USA) metabolic cages were used in the present study to simultaneously assess indirect calorimetry parameters and ambulatory activity in freely moving age-matched control and $Rgs2^{-/-}$ mice under normal and diet-restricted conditions. Here we report that $Rgs2^{-/-}$ mice displayed significantly increased metabolism compared to WT controls, and that these differences were exacerbated both by chronic and by acute caloric restriction. No changes in ambulatory activity were observed, suggesting that internal metabolism, rather than overt physical activity, is the major contributor to the lean $Rgs2^{-/-}$ phenotype. On *ad libitum* diets, although metabolic parameters were observed to decrease over time as mice aged, $Rgs2^{-/-}$ mice displayed consistently higher rates of metabolism compared to WT animals. In combination with our previous findings which extensively evaluated the effects of the loss of RGS2 on various physiological parameters, collectively our data resolve the erstwhile paradox concerning nutrient intake and metabolic rates in $Rgs2^{-/-}$ mice, and demonstrate an important role for RGS2 in the regulation of weight and body metabolism.
Methods

5.2.1 Animals

The generation and genotyping of \( Rgs2^{-/-} \) mice have been previously described (Oliveira-Dos-Santos et al., 2000; Roy et al., 2006). Briefly, \( Rgs2^{-/-} \) mice used for experimental procedures came from congenic \( Rgs2^{-/-} \) and WT control (\( rgs2^{+/+} \)) colonies that are well established in our laboratory (Roy et al., 2006). Prior to CLAMS metabolic cage measurements and diet restriction studies, animals were housed in individual standard shoebox cages and provided \textit{ad libitum} access to standard rodent chow pellets containing 18.6% protein, 6.2% fat, and 44.2% carbohydrates (2018 Teklad global 18% protein diet, Envigo, United Kingdom) and water on a standard 12:12 h light-dark cycle (light period: 0700 – 1900 h). Male \( Rgs2^{-/-} \) and WT controls in C57 Bl/6 backgrounds were used for all studies. Animals were maintained in accordance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. These studies were approved by the Council on Animal Care at the University of Western Ontario, and complied with the guidelines of the Canadian Council on Animal Care.

5.2.2 Study design

Animals were monitored using Oxymax CLAMS metabolic cages (Columbus Instruments, Columbus, OH, USA). During diet studies, individually housed mice were maintained in CLAMS cages at 24 ± 1°C on a standard 12:12 h light-dark cycle (light period: 0700–1900 h) with airflow of 0.35L/min (Guzman et al., 2013). Water was available \textit{ad libitum} in all studies, while powdered standard rodent chow (2018 Teklad) was available depending on the diet study performed. Animals were individually housed in plexiglass cages and were acclimatized to their CLAMS cages for 16-24 hours prior to data acquisition (Guzman et al., 2013).

5.2.3 Assessment of indirect calorimetry and activity

Volume of oxygen consumption (\( VO_2 \)), volume of carbon dioxide production (\( VCO_2 \)), respiratory exchange ratio (RER), cumulative food and water intake, and physical (ambulatory) activity were simultaneously measured using CLAMS and Oxymax software (Columbus Instruments). Measurements for all described parameters were taken every 10 minutes for 24
hours on a standard 12:12 hr light-dark cycle (light period: 0700 – 1900h), and collected data were averaged to produce both one hour time points as well as 12 hour light and dark averages. VO$_2$ and VCO$_2$ were normalized to body mass (mL/kg/hr). Cumulative food and water intake were normalized to 1900 h starting values. RER, an indicator of which macronutrients are being metabolized, was calculated by dividing VCO$_2$/VO$_2$. An RER of ~1.0 or ~0.8 indicates almost complete carbohydrate or fat oxidation, respectively, while values that fall between these extremes suggest a mixture of the energy sources are being metabolized (Richardson, 1929). Energy expenditure (heat) was calculated from RER and VO$_2$, and corrected for body mass (kcal/hr/kg). Ambulatory activity was obtained using Opto-M3 Activity Monitor and Oxymax software algorithms (Columbus Instruments). Infrared photo beam breaks were monitored as counts on the X- and Y-axes; consecutive beam breaks were assessed as ambulatory activity.

5.2.4 Ad libitum studies

1 month, 3 month, and 12 month-old $Rgs2^{-/-}$ and WT controls were placed on ad libitum standard chow diets (2018 Teklad) following weaning. Animals were placed in CLAMS cages for 12 hours of acclimatization followed by 24 hours of data acquisition (start time: 1900 h) on a standard 12:12 h light-dark cycle (light period: 0700 – 1900 h). Ad libitum diet in CLAMS cages was provided in powdered form via a freely accessible center feeder.

5.2.5 Chronic diet restriction studies

Individual food intake for 8 week old $Rgs2^{-/-}$ and WT mice was measured daily for two consecutive weeks to determine average consumption of standard rodent diet (2018 Teklad) for each animal. Based on measured daily amounts, food intake was reduced by 10% every two weeks to reach a final daily diet restriction of 30% after six weeks (i.e. 2 weeks each of 10%, 20%, and 30% reduction); body weights were measured weekly. Daily food allowance was provided in pellet form to all mice at 1100 h. Daily food intake over time was measured by weighing food pellets at 30 min and 1 to 24 hours after introduction of food. During 30% diet restriction, blood glucose levels were measured using a glucometer (OneTouch UltraMini system, Johnson & Johnson, Canada) on tail snip collected blood immediately prior to the
introduction of food, as well as 15 min, 30 min, 1 h, 2 h, and 3 h after the introduction of food. Following two weeks of 30% restriction, at which time mice were 3.5 month (14 weeks) old, animals were placed in CLAMS cages for 12 hours of acclimatization followed by 24 hours of data acquisition (start time: 1900 h), with access to pre-weighed food pellets at 1100 h.

5.2.6 Acute diet restriction studies

3 month-old Rgs2−/− and WT mice on standard ad libitum rodent diet (2018 Teklad) were placed in CLAMS cages for 16 hours of acclimatization followed by 36 hours of data acquisition. Cage feeders with powdered food were replaced with empty feeders for 12 hours (start time: 1900 h), with ad libitum food access returned at 1100 h the following day for another 20 hours (end time: 1900 h).

5.2.7 Microarray analysis

Total RNA was isolated from the gastrocnemius tissue of ad libitum fed 12 week old male Rgs2−/− (n=2) and WT (n=2) mice using the TriZol (Invitrogen) extraction method; RNA integrity was confirmed with Agilent 2100 BioAnalyzer Data Review Software (Agilent, USA) at the London Regional Genomics Centre. Samples with RNA integrity number (RIN) values greater than 8 were used for microarray, which was performed at the London Regional Genomics Centre as previously described (Ratneswaran et al., 2017). Briefly, total RNA was hybridized to the Affymetrix GeneChip® Mouse Gene 2.0 ST Array containing 35 240 probes. Gene level, ANOVA p values, and fold changes were calculated using Partek Genomics Suite version 6.6. Genes with at least a 1.4 fold change and with p<0.05 were used to generate gene ontology biological processes and cellular component processes diagrams through the PANTHER gene list analysis program (http://pantherdb.org/) (Mi et al., 2013). In addition, all genes with p<0.05 were analyzed to identify potential metabolic differences between Rgs2−/− and WT animals.

5.2.8 Quantitative reverse transcriptase qPCR

Total RNA was isolated from the gastrocnemius tissue of ad libitum fed 12 week old male mice using the TriZol (Invitrogen) extraction method. Reverse transcription reaction was
performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Invitrogen, Canada). SensiFAST SYBR No-ROX Kit mastermix (FroggaBio, Canada) was used for real-time thermal cycling. Primers for genes of interest (Table 5.3) were designed using the OligoPerfect Designer (ThermoFisher, Canada), OligoCalc (Kibbe, 2007) and Primer-BLAST (NCBI) programs. Samples were amplified for 35 cycles using a CFX384 real-time thermal cycler (Bio-Rad, Canada). The mRNA quantity for each gene of interest was determined using standard curve analysis and normalized to 28S ribosomal expression.

5.2.9 Statistical analysis

All data were analyzed using GraphPad Prism 6.01 (GraphPad Software, San Diego, CA, USA) and presented as mean ± SEM. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Bonferroni’s post-test and unpaired Student’s two-tailed t-test. Differences were considered significant at P < 0.05.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>serca1</td>
<td>GACGTTTTGTGGTGTGTGTTG</td>
<td>GCAATGGGCTGTACCCCTTGTT</td>
</tr>
<tr>
<td>adcy2</td>
<td>GGAGATCGAAACCATGGAGA</td>
<td>TGGAGGCAAACATGACACA</td>
</tr>
<tr>
<td>adcy5</td>
<td>TCGTGCTGGCTCTGTATCTG</td>
<td>GTCCCTGGGCGAAGATTTGTT</td>
</tr>
<tr>
<td>adrb1</td>
<td>CAGGCGCTGAGTCTCTCTCTCT</td>
<td>TGCCGCTTTGTGTTTTTAGCC</td>
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<tr>
<td>adrb2</td>
<td>AAAGAGCACAAAGCCCTCAA</td>
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</tr>
<tr>
<td>atp5g1</td>
<td>AGGAACCCATCTCTCAAGCA</td>
<td>AGGCGACCATCAAAACAGA</td>
</tr>
<tr>
<td>cs</td>
<td>AGGATCCCATGTCTAAGCTG</td>
<td>TTCATCTCCGTCATGGCCATA</td>
</tr>
<tr>
<td>dio2</td>
<td>GATGCTCCCAATTCCAAGTC</td>
<td>TGAACCAAAAGTTGGACCACCA</td>
</tr>
<tr>
<td>hk2</td>
<td>GGGTAGCCACGGAGTACAAA</td>
<td>TGGATTGAAAGCCCAACTTCC</td>
</tr>
<tr>
<td>pygm</td>
<td>GACATGGAGGAGTTGGAGGA</td>
<td>CATAGCGAAGCGCATACATCA</td>
</tr>
<tr>
<td>ryr1</td>
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<td>TTCCTTCGTCGTCGGCTCTT</td>
</tr>
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<td>GCGGTAAGTGAGTGAGAGGC</td>
</tr>
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<td>slc2a4</td>
<td>GATTCTGGCTGCCCTCTGTG</td>
<td>ATGGACGCTCTCTCTCCAA</td>
</tr>
<tr>
<td>thra</td>
<td>CAAGATCACCAGGAATCATGT</td>
<td>TGCAATGACGCGAAATCATCTC</td>
</tr>
<tr>
<td>ucp3</td>
<td>TCAAGCAGCAGGACTCAGA</td>
<td>GAGAGCAGGAGGAGATTTG</td>
</tr>
</tbody>
</table>
5.3 Results

5.3.1 Increased metabolism over time in $Rgs2^{-/-}$ mice on *ad libitum* diet

To determine the effects of loss of RGS2 on basal metabolism, indirect calorimetry, activity, as well as food and water intake were measured in 1 month, 3 month, and 12 month-old $Rgs2^{-/-}$ and WT mice. In newly weaned 1 month-old $Rgs2^{-/-}$ and WT animals, VO$_2$, VCO$_2$, energy expenditure (heat), ambulatory activity, as well as food and water intake were comparable between phenotypes (Fig. 1A, B, C, D and Fig. 2A and B). Although both groups were more metabolically and physically active during the 12 hour dark cycle (due to the nocturnal nature of mice), there was no significant difference between the described parameters. Interestingly, RER during the 12 hour light cycle was noticeably increased in $Rgs2^{-/-}$ mice (Fig. 1A), suggesting increased carbohydrate metabolism compared to WT controls. Body weights at 1 month were still similar between groups, with significant weight separation observed at the 3 month and 12 month time points (Table 1).

Consistent with our previous observations (Nunn *et al.*, 2011), lean 3 month-old $Rgs2^{-/-}$ mice were found to be hyperphagic in comparison to WT controls (Fig. 2A,), and were also found to consume significantly more water during the 12 hour dark cycle (Figure 2B). If increased metabolism is to account for the lean phenotype of $Rgs2^{-/-}$ mice, it would be expected that both O$_2$ consumption (VO$_2$) and CO$_2$ production (VCO$_2$) would be elevated to some extent. In both strains of mice, these parameters were increased compared to levels during the light cycle, indicative of the nocturnal activity of mice (Fig. 1A and B). However, $Rgs2^{-/-}$ mice displayed additional increases in dark cycle VO$_2$ and VCO$_2$ levels compared to WT mice. Interestingly, significant increases in energy expenditure (heat) between the light and dark cycles were detected only in $Rgs2^{-/-}$ mice (Fig. 1D). RER also appeared to be higher in $Rgs2^{-/-}$ mice at 3 months, again suggesting increased carbohydrate metabolism (Fig. 1C). Similar to 1 month old mice, changes in ambulatory activity were not found between strains, implying that observed metabolic differences do not arise from increased physical activity (Table 1).

Further strain separation of several metabolic parameters was observed in 12 month old animals. VO$_2$, VCO$_2$, and energy expenditure were significantly elevated during the dark cycle.
in Rgs2−/− compared to WT animals (Fig. 1A, B, and D), although food intake was not statistically different at 12 months (Fig. 2A). Patterns in RER (Fig. 1C) and ambulatory activity (Table 1) were similar to those observed for 3 month old mice. Overall, these ad libitum data suggest that Rgs2−/− mice may have a higher basal metabolic demand than WT animals, and that this is maintained throughout their lifespan.

5.3.2 Chronic caloric restriction unmasks metabolic differences between Rgs2−/− and WT mice

Given that some but not all measured parameters pointed to higher metabolic demand in Rgs2−/− mice under normal ad libitum diet conditions and the observed lean phenotype, we reasoned that reducing food intake via a chronic 30% diet restriction might reveal further differences between Rgs2−/− animals and WT controls. Caloric restriction was introduced gradually, with provided daily food reduced by 10% for the first two weeks and then 20% for two subsequent weeks prior to the 30% reduction (Figure 3G). Animals were weighed weekly to ensure they did not drop below acceptable protocol levels. Similar to other diet restriction studies (Weindruch et al., 1986; Faulks et al., 2006), body weights remained essentially constant in both Rgs2−/− and WT mice (Figure 3G). Since WT Bl/6 mice tend to steadily gain weight as they age (Nunn et al., 2011), the lack of such an increase over six weeks indicated that the diet restriction regimen was successful. All indirect calorimetry parameters were significantly altered in Rgs2−/− animals compared to WT controls following 30% diet restriction, with Rgs2−/− mice exhibiting higher levels of VO2, VCO2, RERs, and energy expenditure (heat) (Fig. 3A, B, C, and D). Notably, RER increased substantially in both strains following the introduction of food and reached an elevated plateau, which was significantly higher in Rgs2−/− than WT mice; RER reached and remained at 1.0 or greater in Rgs2−/− mice, a level indicative of energy metabolism based almost entirely on carbohydrate usage (Fig. 3C). Similar to 3 month-old ad libitum studies, physical activity did not appear to play a role in the increased energy expenditure of Rgs2−/− mice (c.f. Tables 1 and 2).

During the 30% diet restriction, the rate of food consumption by Rgs2−/− mice was more than 4 times the rate of the WT diet-restricted mic (Figure 3E). All mice finished their food prior to the next daily feeding, and consequently knockout mice were essentially fasted for
approximately 20 hours before their next meal versus about 12 hours for WT animals, with significantly lower blood glucose levels than WT mice directly prior to food introduction (Figure 3H). Although there were clear strain-related differences evident under caloric restriction, it is possible that the unexpected difference in the duration of fasting might have had a distorting effect on the measurement of metabolic parameters. To control for behavioural differences in food consumption, in further experiments we subjected both WT and KO mice to an acute 16 hour fast and acquired CLAMS data to assess metabolic and physical activity.

5.3.3 Decreased energy conservation in Rgs2−/− mice during and after acute fast

Fasting typically results in energy conservation, which can be demonstrated via decreased VO₂ and VCO₂ levels, and indeed such changes were observed in both WT and Rgs2−/− animals following food removal (Figure 4A and B, respectively), along with a sustained decrease in RER in both strains to a level consistent with primarily fat metabolism (Fig. 4C). During this fasting period, Rgs2−/− mice consumed more oxygen than WT controls (Fig. 4A) indicating a greater metabolic rate. Upon food reintroduction, Rgs2−/− animals shifted from fat metabolism back towards almost complete carbohydrate metabolism (as indicated by RER) more rapidly than their WT counterparts (Fig. 4C), with significant difference between VO₂, VCO₂ and energy expenditure (Fig. 4A, B, and D).

The rapid increases in VO₂, VCO₂, RER, energy expenditure (heat), as well as food and water intake that were observed in our chronic diet restriction Rgs2−/− mice were less pronounced than in our fasting study, suggesting that chronic caloric deprivation and/or prolonged and unequal fasting times may have increased strain-related metabolic differences. Similar to our ad libitum and chronic diet results, there was no discernable difference between genotypes for ambulatory activity (Table 2), suggesting again that weight loss in RGS2 knockout mice is due to internal energy metabolism rather than overt physical activity. Taken together, these findings collectively suggest that Rgs2−/− plays an important role in the regulation of basal metabolism and energy expenditure.
Indirect calorimetry was performed in 1 month, 3 month, and 12 month old mice to measure (A) VO$_2$, and (B) VCO$_2$. White and black bars under time course tracings denote 12 h light and dark cycles, respectively. Bar graphs below time course tracings represent average 12 h light/dark cycles for WT and KO mice. Data represent means ± SEM, n=4, 7-8, and 7 for in 1 month, 3 month, and 12 month old animals, respectively. Data within each graph were analyzed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons, *P<0.05 and **P<0.01.

Figure 5.1. Ad libitum diet, indirect calorimetry, VO$_2$ and VCO$_2$
Indirect calorimetry was performed in 1 month, 3 month, and 12 month old mice to measure (A) energy expenditure (heat). (B) RER was calculated from VCO₂/VO₂. White and black bars under time course tracings denote 12 h light and dark cycles, respectively. Bar graphs below time course tracings represent average 12 h light/dark cycles for WT and KO mice. Data represent means ± SEM, n=4, 7-8, and 7 for in 1 month, 3 month, and 12 month old animals, respectively. Data within each graph were analyzed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons, *P<0.05 and **P<0.01.

Figure 5.2. Ad libitum diet, indirect calorimetry, RER and heat
Figure 5.3. *Ad libitum* diet, food and water intake

(A) Food and (B) water intake were measured simultaneously with indirect calorimetry in CLAMS cages. White and black bars under time course tracings denote 12 h light and dark cycles, respectively. Bar graphs below time course tracings represent average 12h light/dark cycles for WT and KO mice. Data represent means ± SEM, n=4, 7-8, and 7 for in 1 month, 3 month, and 12 month old animals, respectively. Data within each graph were analyzed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons, *P<0.05 and **P<0.01.
Table 5.2. Ambulatory activity and body weights, *ad libitum* diet

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>WT</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>rgs2&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>n</td>
<td>4</td>
<td>8</td>
<td>7</td>
<td>4</td>
<td>8</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BWT (g)</td>
<td>21.0 ± 0.7</td>
<td>26.5 ± 0.4</td>
<td>33.2 ± 0.6</td>
<td>18.2 ± 0.3</td>
<td>24.0 ± 0.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>27.6 ± 0.2&lt;sup&gt;**&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light activity (counts x 10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>6.0 ± 0.3</td>
<td>6.1 ± 1.2</td>
<td>5.7 ± 0.2</td>
<td>7.9 ± 1.0</td>
<td>5.2 ± 0.5</td>
<td>6.4 ± 0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark activity (counts x 10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>32.6 ± 7.7&lt;sup&gt;**&lt;/sup&gt;</td>
<td>19.7 ± 2.5&lt;sup&gt;***&lt;/sup&gt;</td>
<td>23.0 ± 1.9&lt;sup&gt;***&lt;/sup&gt;</td>
<td>23.1 ± 4.3</td>
<td>222 ± 2.1&lt;sup&gt;***&lt;/sup&gt;</td>
<td>18.9 ± 0.8&lt;sup&gt;**&lt;/sup&gt;</td>
<td></td>
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</table>

Abbreviations: BWT, body weight. Ambulatory activity represents counts from x and y axes. Data represent means ± s.e.m. **P<0.05, ***P<0.01 and ****P<0.001, dark vs. light cycle ambulatory activity within genotypes and rgs2<sup>-/-</sup> BWT vs. WT BWT within age groups. Data were analysed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons.
Table 5.3. Ambulatory activity and body weights, chronic and fasting diets

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>rgs2 -/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>30% restriction:</td>
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<tr>
<td>BWT (g)</td>
<td>26.0 ± 0.9</td>
<td>18.8 ± 0.7**</td>
</tr>
<tr>
<td>Light activity (counts x 10³)</td>
<td>9.9 ± 2.1</td>
<td>13.9 ± 0.3</td>
</tr>
<tr>
<td>Dark activity (counts x 10³)</td>
<td>22.1 ± 2.6</td>
<td>17.2 ± 3.1</td>
</tr>
<tr>
<td>16hr fast:</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>BWT (g)</td>
<td>26.8 ± 1.0</td>
<td>23.5 ± 0.9*</td>
</tr>
<tr>
<td>16hr fast activity (counts x 10³)</td>
<td>38.3 ± 1.5</td>
<td>45.8 ± 8.6</td>
</tr>
<tr>
<td>16hr post fast activity (counts x 10³)</td>
<td>16.7 ± 0.4**</td>
<td>19.9 ± 2.7**</td>
</tr>
</tbody>
</table>

Abbreviations: BWT, body weight. Ambulatory activity represents combined counts from x and y axes. Data represent means ± s.e.m. Body weights were compared using Student’s t-test. Ambulatory activities were compared using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons, dark vs. light cycle within genotypes and 16 h fast vs. 16 h post-fast within genotypes, **P<0.05, ***P<0.001.
Figure 5.4. Chronic diet restriction, indirect calorimetry

3 month old male $\text{Rgs2}^{-/-}$ and WT mice were subjected to 30% chronic diet restriction following 2 weeks each of 10% and 20% restriction. Indirect calorimetry was performed to measure (A) VO$_{2}$, (B) VCO$_{2}$, and (D) energy expenditure (heat), which were normalized to body weight. (C) RER was calculated from VCO$_{2}$/VO$_{2}$. White and black bars under time course tracings denote 12 h light and dark cycles, respectively. Bar graphs below time course tracings represent average 12 h light/dark cycles for WT and KO mice. Data represent means ± SEM, n=4 per group. Data in individual graphs were analyzed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons, *P<0.05, **P<0.01 and ***P<0.001.
Figure 5.5. Chronic diet restriction

(A) Food and (B) water intake were measured simultaneously with indirect calorimetry in CLAMS cages. (C) Body weights were recorded weekly to monitor effects of 10%, 20% and 30% caloric restriction. (D) Glucose levels under 30% caloric restriction were recorded immediately prior and 15 min, 30 min, 1 h, 2 h, and 3 h post-feeding. White and black bars under time course tracings denote 12 h light and dark cycles, respectively. Bar graphs below time course tracings represent average 12 h light/dark cycles for WT and KO mice. Data represent means ± SEM, n=4 per group. Data in individual graphs were analyzed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons, *P<0.05, **P<0.01 and ***P<0.001.
Figure 5.6. Acute 16 h fast, indirect calorimetry

3 month old male Rgs2^-/- and WT mice were subjected to a 16 h fast followed by reintroduction of ad libitum diet. Indirect calorimetry was performed to measure (A) VO2 and (B) VCO2. White and black bars under time course tracings denote 12 h light and dark cycles, respectively. Bar graphs below time course tracings represent average 12h light/dark cycles for WT and KO mice. Data represent means ± SEM, n=11 per group. Data within each graph were analyzed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons, *P<0.05, **P<0.01 and ***P<0.001.
Figure 5.7. Acute 16 h fast, indirect calorimetry

(A) energy expenditure (heat), was normalized to body weight, and (B) RER was calculated from VCO₂/VO₂. White and black bars under time course tracings denote 12 h light and dark cycles, respectively. Bar graphs below time course tracings represent average 12h light/dark cycles for WT and KO mice. Data represent means ± SEM, n=11 per group. Data within each graph were analyzed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons, *P<0.05, **P<0.01 and ***P<0.001.
Figure 5.8. 16 h fast, food and water intake

(A) Food and (B) water intake were measured simultaneously with indirect calorimetry in CLAMS cages, and normalized to body weight. White and black bars under time course tracings denote 12 h light and dark cycles, respectively. Bar graphs below time course tracings represent average 12h light/dark cycles for WT and KO mice. Data represent means ± SEM, n=11 per group. Data within each graph were analyzed using two-way ANOVA with Bonferroni’s post hoc test for multiple comparisons, *P<0.05, **P<0.01 and ***P<0.001.
5.3.4 Altered expression of metabolic genes in *Rgs2*⁻/⁻ mice

Genes involved with calcium signalling and metabolism were examined in gastrocnemius tissue to determine potential differences in expression between *Rgs2*⁻/⁻ and WT mice on an *ad libitum* diet. Metabolic genes to analyze with qPCR were chosen based on reviews of the current literature involving skeletal tissue metabolism (Salvatore *et al.*, 2013), and purported expression changes from the performed microarray. A majority of genes reported to be significantly different or unchanged in the micro array could not be validated by qPCR (Suppl. Table C2); thus, microarray data has not been included in the results or discussion in this chapter, but can be viewed (along with PANTHER analysis of biological and cellular processes) in the Appendix (Suppl. Table C2, Suppl. Fig. C2 and C3).

Following qPCR of selected genes, ryanodine1 (*ryr1*), glycogen phosphorylase (*pygm*), and glucose transporter 4 (*Glut4/Slc2a4*) gene expression were all significantly increased in *Rgs2*⁻/⁻ mice compared to WTs (Fig. 5.9B, E, and F). Furthermore, deiodinase type 2 (*Dio2*) expression was significantly enhanced in *Rgs2*⁻/⁻ mice (Fig. 5.10A). Gene expression for sercal (*Atp2a1*), hexokinase 2 (*Hk2*), citrate synthase (*Cs*), thyroid hormone receptor alpha (*Thra*), thyroid hormone-specific transporter MCT8 (*Slc16a2*), ATP synthase subunit F(0) complex subunit C1 (*Atp5g1*), mitochondrial uncoupling protein 3 (*Ucp3*), β₁ and β₂-adrenergic receptors, and adenylyl cyclase 2 and 5 were not found to be significantly altered in *Rgs2*⁻/⁻ mice compared to WT animals (Fig. 5.9, 5.10, and 5.11).
Figure 5.9. Gene expression, calcium signalling and metabolic markers

(A) Serca2a (Atp2a1), (B) ryanodine receptor type 1 (Ryr1), (C) citrate synthase (Cs), (D) hexokinase II (Hk2), (E) glycogen phosphorylase (Pygm), and (F) glut-4 (Slc2a4) gene expression was assessed by qPCR in gastrocnemius tissue of Rgs2−/− and WT mice. Data represent means ± SEM, n=4-6 per group. Data were analyzed by Student’s t-test, *P<0.05.
Figure 5.10. Gene expression, metabolic markers

(A) Deiodinase type 2 (Dio2), (B) thyroid hormone receptor alpha (Thra) and (C) thyroid hormone-specific transporter MCT8 (Slc16a2), (D) ATP synthase subunit F (0) complex subunit C1 (Atp5g1), and (E) mitochondrial uncoupling protein 3 (Ucp3) gene expression was assessed by qPCR in gastrocnemius tissue of Rgs2−/− and WT mice. Data represent means ± SEM, n=4-6 per group. Data were analyzed by Student’s t-test, *P<0.05.
Figure 5.11. GPCR-related gene expression

(A) beta-1 adrenergic receptor (*Adrb1*), (B) beta 2 adrenergic receptor (*Adrb2*), (C) adenylyl cyclase 2 (*Adcy2*), and (D) adenylyl cyclase 5 (*Adcy5*) gene expression was assessed by qPCR in gastrocnemius tissue of *Rgs2<sup>−/−</sup>* and WT mice. Data represent means ± SEM, n=4-6 per group. Data were analyzed by Student’s t-test.
5.4 Discussion

The present study expands on our previous work, in which we found Rgs2<sup>−/−</sup> mice to be resistant to normal, age-related weight gain (Nunn et al., 2011). This observed decrease in body weight was also accompanied by increased insulin sensitivity, smaller adipocytes, decreased adipose tissue deposits, and lower levels of serum lipids. In addition, nutrient absorption was comparable to WT controls (Nunn et al., 2011). We were previously unable to reconcile obvious changes in lipid metabolism and body weight with measured metabolic rates; using the more sensitive CLAMS cages, we demonstrate in this study that metabolic rates as measured by indirect calorimetry are increased in Rgs2<sup>−/−</sup> mice. These effects are further enhanced by both chronic and acute diet restrictions, and also suggest that Rgs2<sup>−/−</sup> mice may have enhanced carbohydrate metabolism. To our knowledge, we are the first group to report a metabolic phenotype in Rgs2<sup>−/−</sup> mice.

Our ad libitum studies demonstrate that basal metabolic indices in 3 month and 12 month old Rgs2<sup>−/−</sup> mice were elevated compared to WT controls. A lack of observable differences in VO<sub>2</sub>, VCO<sub>2</sub>, and energy expenditure at the 1 month time point may be age related; at this stage, both WT and Rgs2<sup>−/−</sup> animals are still in a rapid growth phase (Somerville et al., 2004), which could obscure the slight differences seen in the older age groups. When viewed collectively over time, VO<sub>2</sub> and VCO<sub>2</sub> values do fall with increasing age (Figure 1A and B), which is expected as body metabolism slows (LeBrasseur et al., 2009). However, Rgs2<sup>−/−</sup> mice consistently displayed higher VO<sub>2</sub> and VCO<sub>2</sub> levels compared to WT controls (Fig. 1A and B). Since changes in the 2-5% range can induce significant long term alterations in energy balance (Even and Nadkarni, 2012), the small, but sustained elevations in VO<sub>2</sub> and VCO<sub>2</sub>, particularly during dark cycles, may contribute to the observed lean phenotype.

In our previous studies, we determined that although Rgs2<sup>−/−</sup> mice had reduced fat stores and smaller adipocytes, they were not resistant to high fat diet-induced obesity, and developed insulin resistance to a similar degree as WT mice (Nunn et al., 2011). This suggested that Rgs2<sup>−/−</sup> mice do not have major impairments with respect to their ability to accumulate adipose tissue, and that alterations to carbohydrate or protein rather than fat metabolism could be additional factors in the development of the lean phenotype. Since our ad libitum RER calculations...
suggested that carbohydrate metabolism was slightly increased in \textit{Rgs2}^{-/-} mice (Fig. 1C), we challenged adult, 3 month-old animals to a chronic 30\% diet restriction to further evaluate metabolic parameters. Both strains did not lose substantial body weight (Fig. 3G); final body weights after two weeks of 30\% diet restriction (which was also preceded by two weeks each of 10\% and 20\% restrictions) were approximately 3\% and 6\% higher in \textit{Rgs2}^{-/-} and WT controls compared to initial starting \textit{ad libitum} body weights, respectively. Although this suggested that a 30\% restriction can be handled by both strains, and that \textit{ad libitum} diet well exceeds necessary caloric requirements, \textit{Rgs2}^{-/-} feeding behaviour rapidly changed on the restricted diet, such that hyperphagia was exacerbated to such an extent that feeding and fasting times were significantly altered compared to control mice. RER recordings indicated that upon food consumption, KO mice quickly reached an almost full carbohydrate metabolizing state (RER around 1.0) versus WT mice who continued to demonstrate some fat metabolism (RER around 0.9) (Fig. 3C). Even though increased fasting length due to rapid food consumption may have affected indirect calorimetry readings, the differences between strains is still a relevant observation. VO$_2$, VCO$_2$, RER, and energy expenditure (heat) parameters rapidly increased and were sustained at elevated levels for several hours during and after food consumption; these rapid shifts in metabolism were not observed in WT controls (Fig. 3A, B, C, and D).

To rule out potential confounding effects of the unexpected strain-related difference in food consumption patterns in our chronic restriction studies, food intake was controlled via 16 hour acute fasting studies (Fig. 4). Overall, strain-related differences were of a similar nature under both caloric restriction protocols, albeit less pronounced under acute conditions. Indirect calorimetry recordings during the acute fasting period furthermore suggested a delay in the ability of \textit{Rgs2}^{-/-} mice to lower their metabolism (for energy conservation) compared to WT animals. Conversely, it appears that \textit{Rgs2}^{-/-} mice are able to shift to carbohydrate metabolism in the presence of food at a faster rate than WT mice, as evidenced by higher RERs (Fig. 4C), although it is possible that due to their decreased adiposity, increased carbohydrate metabolism is a compensatory, rather than causative response of the observed lean phenotype (Sun \textit{et al.}, 2012).
Since physical activity was found to be similar across strains, our metabolic parameters across multiple diet modalities suggest that decreased body weights in Rgs2\(^{-/-}\) mice arise instead from internal metabolism. qPCR analysis of gastrocnemius tissue suggests that several metabolically-relevant genes may be altered in Rgs2\(^{-/-}\) compared to WT mice. Skeletal ryanodine receptor type 1 (Ryr1) mediates calcium release; as the main ion channel in the sarcoplasmic reticulum, it is a key determinant of muscle force (Beard, Wei and Dulhunty, 2009). Cells overexpressing Ryr1 have been shown to have higher mitochondrial ATP concentrations, as well as increased ATP production in response to cytosolic Ca\(^{2+}\) elevations. Thus, the observed increases in Ryr1 gene expression in Rgs2\(^{-/-}\) mice could indicate increased levels of ATP production, and indirectly suggest increased ATP utilization in skeletal tissue, which could contribute to the lean phenotype. In addition, increased Rgs2\(^{-/-}\) gene expression of glycogen phosphorylase (Pygm) (the rate-limiting enzyme of glycogenolysis) (Fig. 5.9D) correlates with previous studies which have demonstrated that cultured human muscle cells overexpressing glycogen phosphorylase display increased glucose transporter (GLUT-4) (slc2a4) expression with no changes in hexokinase II (the rate limiting enzyme in glycolysis) (Baqué et al., 1998), expression patterns which we also observed in Rgs2\(^{-/-}\) mice compared to WT controls (Fig. 5.9C and E).

Changes in Dio2 gene expression also suggest increased metabolism in Rgs2\(^{-/-}\) mice (Fig. 5.10A). Since DIO2 catalyzes the conversion of the inactive prohormone thyroxine (3,5,3',5'-tetraiodothyronine, T4) to the active thyroid hormone (3,5,3'-triiodothyronine, T3) (Gereben et al., 2008; Arrojo e Drigo et al., 2013), it would follow that local cellular production of T3 could increase due to elevated levels of DIO2. Thyroid hormone plays a major role in energy expenditure and glucose homeostasis; thyroid hormone signalling can modulate metabolic rate by either uncoupling the ATP synthesis in the mitochondria or decreasing the metabolic efficiency of skeletal muscle (Salvatore et al., 2013), effectively “wasting” energy as dissipated heat (de Lange et al., 2001; Silva, 2006). In addition, GLUT-4 expression (and therefore glucose uptake in cells) is T3 dependent; in conjunction, GLUT-4 translocation to the cell surface is an insulin-dependent event, and normal muscle responsiveness to insulin has been shown to require the DIO2-mediated conversion of T4 to T3 (Mills et al., 1987; Zorzano, Palacin and Guma, 2005; Grozovsky et al., 2009). Altogether, the observed increases in gene
expression for Rylr1, Pygm, Slic2a4 (GLUT-4), and Dio2 suggest that both glucose metabolism and resting metabolic rate may be altered in Rgs2−/− mice compared to WT animals.

Although the exact mechanisms behind the lean phenotype are yet to be determined, RGS2 has been shown to have various effects on energy storage and usage; in vitro studies have demonstrated the role of RGS2 in mediating GIP (glucose-dependent insulinotropic polypeptide)-induced insulin release from pancreatic β cells (Tseng and Zhang, 1998), as well as GLUT-4 (insulin-responsive glucose transporter) translocation in adipocytes (Imamura et al., 1999). Thus, RGS2 may be involved in glucose entrance in adipocytes. RGS2 is also transiently and rapidly upregulated in differentiating mouse preadipocyte cells, and appears to promote the differentiation of adipocytes and expression of adipogenic markers (Imagawa, Tsuchiya and Nishihara, 1999; Nishizuka et al., 2001). Other energy-dependent processes affected by RGS2 include the regulation of protein synthesis (Nguyen et al., 2009; Chidiac et al., 2014), as well as ATP hydrolysis by PMCA (plasma membrane Ca2+ ATPase) and SERCA (sarco/endoplasmic reticulum Ca2+) pumps (Wang et al., 2004). Human polymorphisms which result in the over or under expression of RGS2 have demonstrated the clinical relevance of RGS2 in the context of metabolism. A common polymorphism in the 3’ untranslated region of RGS2 has been shown to decrease RGS2 expression, has been associated with hypertension, and is considered to be a genetic marker that can predict male predisposition to weight gain and obesity (Sartori et al., 2008). Conversely, a C to G substitution in the RGS2 promoter region has been shown to increase RGS2 expression in adipocytes and is associated with the metabolic syndrome (Freson et al., 2007). Altogether, our current findings suggest an important role for RGS2 in weight regulation and body metabolism.

5.5 Conclusions

Using simultaneous measurements of indirect calorimetry and activity, we have demonstrated increased metabolism in Rgs2−/− mice compared to WT controls in both ad libitum and diet restriction studies. Obesity is a common, costly, and serious disease, with more than 2/3 of US adults considered to be overweight or obese (Flegal et al., 2012). This growing epidemic makes
the study of potential regulators of metabolic signaling both timely and necessary. Given that increased RGS2 expression may be involved in obesity and metabolic disease in humans, and our findings that a lack of RGS2 leads to decreased body weight and improved metabolic profiles, RGS2 may be an important target for the future development of anti-obesity strategies.
References


de Lange, P., Lanni, A., Beneduce, L., Moreno, M., Lombardi, A., Silvestri, E. and Goglia, F.


6 DISCUSSION AND CONCLUSIONS
6.1 Summary and Discussion

6.1.1 Chapter 3 – Cardiomyocyte specific overexpression of a 37 amino acid domain of RGS2 inhibits cardiac hypertrophy and improves function in response to pressure overload in mice

Cardiac hypertrophy is a known risk factor for cardiovascular diseases such as heart failure, and involves de novo protein synthesis in the post-mitotic cardiomyocyte population of the heart (Anversa et al. 2007; Carvalho 2010). RGS2 has been well established as an inhibitor of cardiomyocyte hypertrophy via its inhibitory effects on G\textsubscript{q} and G\textsubscript{s}-mediated signalling (Roy et al. 2006; Zou et al. 2006; Nunn et al. 2010; Zhang et al. 2011). We have previously reported that RGS2 can also inhibit protein synthesis through a 37 amino acid domain within its RGS domain (Nguyen et al. 2009), which we have termed RGS2\textsuperscript{eb}. We observed these effects in both cell lines and cultured neonatal cardiomyocytes (Chidiac et al. 2014; Nguyen et al. 2009), and thus hypothesized that the cardiomyocyte-specific overexpression of RGS2\textsuperscript{eb} could result in the in vivo inhibition of protein synthesis, and potentially offer protection against the development of hypertrophy in an experimental model of pressure overload. The aim of the studies described in Chapter 3 of this thesis was to determine the extent of cardiac hypertrophy in transgenic RGS2\textsuperscript{eb} (RGS2\textsuperscript{eb} TG) mice compared to WT sham and transverse aortic constriction (TAC) animals, and whether there were associated benefits to cardiovascular function.

Following 28 days of TAC, mice with cardiomyocyte specific overexpression of RGS2\textsuperscript{eb} were resistant to the development of significant cardiac hypertrophy compared to WT TAC mice. At the cellular level, measurements of cardiomyocyte cross-sectional area showed a corresponding increase in cell size for WT but not RGS2\textsuperscript{eb} TG mice after TAC. In addition, systolic and diastolic indices of cardiac function via pressure-volume loop analyses suggested that RGS2\textsuperscript{eb} TG animals were able to maintain significantly improved cardiac function following TAC compared to WT animals. Furthermore, the expression of the “fetal” genes β-MHC and ANP, which are often upregulated during conditions of hypertrophy and heart failure, was completely abrogated in RGS2\textsuperscript{eb} TG compared to WT TAC mice. These results
are consistent with our overall hypothesis that expression of the eIF2B-interacting domain of RGS2, RGS2\textsuperscript{eb}, can protect against pathological cardiac hypertrophy and dysfunction \textit{in vivo}, and that this may be via the direct inhibition of protein synthesis. Along with our previous studies, which showed the ability of RGS2\textsuperscript{eb} to inhibit drug-induced cardiomyocyte hypertrophy, our current findings suggest that the RGS2\textsuperscript{eb} region is an important contributor to the reported beneficial cardiac effects of RGS2.

**6.1.2 Chapter 4 – Effects of transverse aortic constriction and drug induced experimental pressure overload hypertrophy on Rgs2\textsuperscript{−/−} mice with cardiomyocyte specific overexpression of RGS2\textsuperscript{eb}**

The aim of Chapter 4 was to explore whether the cardioprotective effects of RGS2\textsuperscript{eb} that we observed in Chapter 3 could be extended to an Rgs2\textsuperscript{−/−} model of experimentally-induced cardiac hypertrophy. Rgs2\textsuperscript{−/−} mice are highly sensitive to TAC-induced cardiac hypertrophy and heart failure (Takimoto et al. 2009), and have been shown to have mild to moderate levels of hypertension (Oliveira-Dos-Santos et al. 2000; Heximer et al. 2003). The use of Rgs2\textsuperscript{−/−} mice could provide further clarity into the cardioprotective role of the RGS2\textsuperscript{eb} region, endogenous RGS2 was still present in the Rgs2\textsuperscript{+/+} (eb) mice from our earlier studies (Lee et al. 2017). Based on our observations in Chapter 3, we hypothesized that the cardiomyocyte specific expression of RGS2\textsuperscript{eb} in Rgs2\textsuperscript{−/−} mice (Rgs2\textsuperscript{−/−}(eb)) could potentially decrease the severity of cardiac hypertrophy and heart failure resulting from experimentally induced pressure overload, thereby compensating for the absence of full-length RGS2.

For our Chapter 4 studies, we generated novel transgenic Rgs2\textsuperscript{−/−} mice with cardiomyocyte specific overexpression of RGS2\textsuperscript{eb} (Rgs2\textsuperscript{−/−}(eb)). However, when we initially began our TAC studies with Rgs2\textsuperscript{−/−} and WT mice, we found that the mortality rate for Rgs2\textsuperscript{−/−} animals significantly exceeded that of WT controls following TAC surgery, suggesting an impaired ability to recover from cardiovascular insult. Due to the unexpectedly high mortality rate of Rgs2\textsuperscript{−/−} mice, the TAC model was determined to be unsuitable for the evaluation of cardiac hypertrophy, and was discontinued before the addition of Rgs2\textsuperscript{−/−}(eb) mice to the study. We thus proceeded to examine the effects of drug-induced models of hypertension on Rgs2\textsuperscript{−/−} mice. Starting with an angiotensin II model of chronic agonist infusion \textit{via} osmotic pumps
(1.44mg/kg/day), we observed that although there was hypertension in our angiotensin II treated animals, significantly detectable cardiac hypertrophy did not develop in any experimental angiotensin II groups (WT, Rgs2<sup>+/+<sup>(eb), Rgs2<sup>-/-, and Rgs2<sup>-/-<sup>(eb)) compared to saline control mice. Further studies with angiotensin II infusion at higher concentrations (3.0 mg/kg/day for 14 days) using WT and Rgs2<sup>-/- mice also failed to induce clearly detectable cardiac hypertrophy. Thus, we went on to perform pilot studies with twice daily injections of isoproterenol (ISO) for 14 days; our preliminary results suggest that a full study with prolonged injections beyond 14 days may result in cardiac hypertrophy. Such a reproducible model of cardiac hypertrophy would then allow for the effective examination of whether the inhibitory effects of RGS2<sup>eb on protein translation is sufficient to provide cardioprotection in a null RGS2 background.

6.1.3 Chapter 5 – Loss of RGS2 alters the effects of acute and chronic diet restriction on metabolic outcomes in C57 BL.6 mice

Cardiovascular dysfunction, including pathological cardiac hypertrophy, is known to be directly influenced by obesity and weight gain, which are major risk factors for cardiovascular diseases such as coronary heart disease, atrial fibrillation, hypertension, venous thromboembolisms, and congestive heart failure (Klein et al. 2004; Poirier et al. 2006; Tedrow et al. 2010; Wattanakit et al. 2012). Adiposity is determined by energy expenditure versus energy intake, and possible sources of energy imbalance in cases of obesity or weight loss could come from the dysregulation of food intake/nutrient absorption, lipid storage, metabolic rate, physical activity, or CNS-related changes.

Multiple GPCRs have been shown to be involved in energy metabolism, and alterations in G protein signalling (which is regulated by RGS proteins) can lead to both lean and obese phenotypes (Colombo et al. 1998; Nambu et al. 2011; Grillet et al. 2005; Cho et al. 2008). We have previously reported that Rgs2<sup>-/- mice exhibit a lean phenotype, are hyperphagic, have smaller adipose tissue deposits and adipocytes, and are ultimately resistant to age-related weight gain (Nunn et al. 2011). In addition, comparable nutrient absorption suggested that increased physical activity and/or metabolism could be underlying factors to the observed lean phenotype. However, although the lean phenotype was present throughout the Rgs2<sup>-/- mouse
lifespan on an *ad libitum* standard rodent diet, these animals nonetheless became obese and insulin-resistant under high-fat diet conditions (Nunn et al. 2011), suggesting that *Rgs2*<sup>-/-</sup> mice do not have a major impairment with respect to their ability to accumulate adipose. Thus, we hypothesized that the observed lean phenotype may reflect underlying changes in energy utilization. The aim of our studies in Chapter 4 was to understand the role of RGS2 in metabolism and body weight regulation using CLAMS metabolic cages to simultaneously assess indirect calorimetry parameters and ambulatory activity in freely moving age-matched control and *Rgs2*<sup>-/-</sup> mice under normal and diet-restricted conditions. Analysis of *ad libitum* data showed *Rgs2*<sup>-/-</sup> mice had significantly increased metabolism compared to WT controls, and that these differences were exacerbated both by chronic and acute caloric restriction. Since no changes in ambulatory activity were observed, these observations suggest that internal metabolism, rather than overt physical activity, is the major contributor to the lean *Rgs2*<sup>-/-</sup> phenotype. Furthermore, since skeletal muscle is a major site of metabolism (Salvatore et al. 2013) qPCR for metabolically-related genes in the gastrocnemius tissue of WT and *Rgs2*<sup>-/-</sup> mice suggest that both glucose metabolism and resting metabolic rate may be altered in *Rgs2*<sup>-/-</sup> mice compared to WT animals. Altogether, our current findings in combination with our previous reports demonstrate an important role for RGS2 in the regulation of weight and body metabolism.

### 6.2 Future directions

In Chapter 3, we demonstrated the cardioprotective potential of RGS2<sub>eh</sub> in an *in vivo* model of cardiac hypertrophy. Currently, the majority of drugs for cardiovascular diseases target GPCRs (Rask-Andersen, Almén, and Schiöth 2011). Although these medications can offer significant benefits to patients, heart failure continues to be a global leading cause of morbidity and mortality. In addition, inhibiting GPCR signalling often results in “off-target” effects due to the broad cascade of signalling pathways that they control (Allen and Roth 2011). The specificity of RGS2<sub>eh</sub> for the inhibition of protein synthesis at the level of translation initiation could potentially mitigate such issues and lead to improved therapeutics. Indeed, interest in RGS2 and other RGS proteins as possible drug targets is on the rise (Sjogren et al. 2012;
Future steps in characterizing the potential of RGS2^eb as a cardioprotective factor could involve its direct delivery to the heart after experimentally-induced cardiac injury in lieu of our current cardiac-specific overexpression model, where activation of the RGS2^eb transgene in the heart occurs soon after birth. Recombinant adeno-associated viruses (rAAVs) could be a potential avenue for RGS2^eb delivery to the heart, as they are currently the most widely used vectors for safe, long-term gene delivery and expression in the heart due to their ability to transduce post-mitotic cells such as cardiomyocytes (Zsebo et al. 2014). rAAVs are unique among viruses used for gene therapy in that the wild-type (AAV) virus has not been shown to cause human disease (Thomas, Ehrhardt, and Kay 2003). In addition to their low immunogenicity, rAAVs demonstrate efficient long-term transgene expression and low rates of insertional mutagenesis; previous experiments using various combinations of AAV capsids (which control receptor-mediated cellular entry) and rAAV pseudotypes have established rAAV2/1, rAAV2/6, rAAV2/8, rAAV2/9, and rAAV2/10 as the most cardiotropic (Pacak and Byrne 2011). Multiple in vivo studies and clinical trials lend credence to the use of rAAVs as a delivery system for RGS2^eb to the heart (Zsebo et al. 2014; Li et al. 2003; Hoshijima et al. 2002; Asfour et al. 2002; Kawase et al. 2008; McTiernan et al. 2007), which could be explored in future studies.

In Chapter 4, the substantially lower survival rates of Rgs2^−/− mice compared to WT counterparts following TAC surgery support studies in the literature that suggest that impaired cardiovascular function in Rgs2^−/− mice may be due to enhanced susceptibility to atrial fibrillation (Jones, Tuomi, and Chidiac 2012). Although our specific aims and hypotheses for this chapter focused on cardiac hypertrophy and related functional effects, the observed Rgs2^−/− mortality rates suggest that further exploration of this reaction to TAC surgery could prove interesting. In terms of understanding any potential roles of RGS2^eb in Rgs2^−/− mice, protocol optimization is certainly required for our studies. Although cardiac hypertrophy was not induced in our experimental angiotensin II animals, the presence of hypertension reflected successful infusion of the drug into agonist-treated mice. Since hypertension-induced hypertrophy via angiotensin II is a less severe model of hypertrophy compared to TAC surgeries, prolonged angiotensin II infusion beyond our current 28 day study design may
clarify the timeline for cardiac hypertrophy development in our mouse strains; similar studies would extend to our pilot isoproterenol models.

The Chapter 5 studies expand on our previous work, in which we found $Rgs2^{-/-}$ mice to be resistant to normal, age-related weight gain (Nunn et al. 2011). We were previously unable to reconcile obvious changes in lipid metabolism and body weight with measured lower metabolic rates; using the more sensitive CLAMS cages, we demonstrate in this study that metabolic rates as measured by indirect calorimetry are in actuality increased in $Rgs2^{-/-}$ mice. These effects are further enhanced by both chronic and acute diet restrictions, and also suggest that $Rgs2^{-/-}$ mice may have enhanced carbohydrate metabolism. To our knowledge, we are the first group to report a metabolic phenotype in $Rgs2^{-/-}$ mice. Future studies to fully characterize this phenotype could examine the effects of various physical activity modalities; since skeletal muscle is one of the most metabolically active tissues, and we observed the upregulation of several genes which regulate glucose and basal metabolism, studies using physical activity as a variable (e.g. treadmill running) could prove interesting. Furthermore, a detailed assessment of additional metabolic genes (perhaps via RNA-seq analysis) is warranted given the currently observed effects of $Dio2$, $Glut-4$, $Ryr1$, and $Pygm$ gene expression, and could ultimately lead to a clearer understanding of the specific mechanisms and key GPCRs which drive the $Rgs2^{-/-}$ lean phenotype.

6.3 Conclusions

Cardiovascular diseases and metabolic dysregulation affect millions around the world, and the study of their underlying factors and mechanisms could lead to the identification of novel drug targets for improved treatment. The first part of this thesis examined the cardioprotective potential of RGS2$^{eb}$, and we determined that cardiac hypertrophy could indeed be inhibited following TAC by the targeted expression of this truncated protein, with accompanying improvements in cardiac function. Although we were limited in our characterization of the $Rgs2^{-/-(eb)}$ mouse line in the second part of our thesis, the poor survival rates that we observed in $Rgs2^{-/-}$ animals following TAC surgery may lead to additional studies examining RGS2
activity in the context of cardiac electrophysiology. The metabolic role of RGS2 is explored in the third part of this thesis, and reveals that loss of RGS2 leads to significant upregulation in metabolic genes and basal metabolism. Altogether, these studies provide new insights into the role of RGS2 in cardiovascular health and metabolism.
6.4 References


Zsebo, K., Yaroshinsky, A., Rudy, J. J., Wagner, K., Greenberg, B., Jessup, M. and Hajjar, R.
APPENDICES
Appendix A: Ethics approval

AUP Number: 2015-079
PI Name: Chidiac, Peter
AUP Title: Understanding Rgs Proteins

Approval Date: 02/16/2016

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled “Understanding Rgs Proteins” has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, is subject to annual Protocol Renewal.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care
AUP Number: 2015-079
PI Name: Chidiac, Peter
AUP Title: Understanding RGS Proteins

Official Notification of AUS Approval: A MODIFICATION to Animal Use Protocol 2015-079 has been approved.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee
Appendix B: Copyright approval

Title: Cardiomyocyte specific overexpression of a 37 amino acid domain of regulator of G protein signalling 2 inhibits cardiac hypertrophy and improves function in response to pressure overload in mice

Author: Katherine N. Lee, Xiangru Lu, Chau Nguyen, Qingping Feng, Peter Chidiac

Publication: Journal of Molecular and Cellular Cardiology

Publisher: Elsevier

Date: July 2017

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Appendix C: Supplementary information

Table C1. Measurement and calculation definitions for VisualSonics analysis of cardiac function

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<td>mm</td>
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<td>LVAW;s</td>
<td>Left ventricular anterior wall (systole)</td>
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<td>Left ventricle volume systole</td>
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<td>%</td>
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<td>%FS (M-Mode)</td>
<td>LV fractional shortening</td>
<td>%</td>
<td>100 * ((LVID;d – LVID;s) / LVID;d)</td>
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<td>LV Mass (M-Mode)</td>
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<tr>
<td>LV Mass Cor (M-Mode)</td>
<td>LV mass corrected</td>
<td>mg</td>
<td>LV Mass (M-Mode) \times 0.8</td>
</tr>
</tbody>
</table>
A

B

C

D

**Figure C1. Body weights and survival, 14 days of twice daily saline or 50 mg/kg ISO**

14 day (A) Saline and (B) ISO-treated mice were weighed daily, with (C)(D) survival recorded for each treatment and genotype. Data represent means ± SEM, n=3-8 per group using two way ANOVA with Bonferroni’s post hoc test for body weight data and Log rank test for survival curves.
Table C2. Selected microarray genes, fold change, and significance

Of the genes below, only *Dio2* expression and significance were validated by qPCR. Gastrocnemius tissue, n=2 per group (WT and KO).

<table>
<thead>
<tr>
<th>Gene</th>
<th>p-value</th>
<th>fold change</th>
<th>fold change description</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Adcy2</em></td>
<td>0.380</td>
<td>3.428</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Adcy5</em></td>
<td>0.001</td>
<td>-1.478</td>
<td>KO down vs WT</td>
</tr>
<tr>
<td><em>Adrb1</em></td>
<td>0.011</td>
<td>1.239</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Adrb2</em></td>
<td>0.507</td>
<td>1.785</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Atp2a1</em></td>
<td>0.414</td>
<td>17.464</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Atp5g1</em></td>
<td>0.074</td>
<td>1.434</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Cs</em></td>
<td>0.287</td>
<td>1.930</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Dio2</em></td>
<td>0.036</td>
<td>1.343</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Hk2</em></td>
<td>0.355</td>
<td>5.699</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Pygm</em></td>
<td>0.419</td>
<td>20.016</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Ryr1</em></td>
<td>0.430</td>
<td>11.336</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Slc16a2</em></td>
<td>0.440</td>
<td>-3.300</td>
<td>KO down vs WT</td>
</tr>
<tr>
<td><em>Slc2a4</em></td>
<td>0.378</td>
<td>7.765</td>
<td>KO up vs WT</td>
</tr>
<tr>
<td><em>Thra</em></td>
<td>0.477</td>
<td>1.402</td>
<td>KO up vs WT</td>
</tr>
</tbody>
</table>
Figure C2. Biological processes, Rgs2$^{-/-}$ down vs. WT

(A) Microarray genes with a higher expression in Rgs2$^{-/-}$ mice vs. WT (1.4 fold or higher and P<0.05) were analyzed by the PANTHER gene list program for functional classifications. (B) The metabolic process section was further expanded for secondary level process details. Chart legends are read as: category name (accession) (# genes; percent of gene hit against total # process hits).
Figure C3. Biological processes, $Rgs2^{-/-}$ up vs. WT

(A) Microarray genes with a lower expression in $Rgs2^{-/-}$ mice vs. WT (1.4 fold or higher and $P<0.05$) were analyzed by the PANTHER gene list program for functional classifications. (B) The metabolic process section was further expanded for secondary level process details. Chart legends are read as: category name (accession) (# genes; percent of gene hit against total # process hits).
Figure C4. Cellular components and molecular function for microarray genes, Rgs2−/− genes up or down vs. WT

(A) Microarray genes with a higher expression in Rgs2−/− mice vs. WT (1.4 fold or higher and P<0.05) were analyzed by the PANTHER gene list program for cellular components and molecular function, as were (B) genes with lower expression. Chart legends are read as: category name (accession) (# genes; percent of gene hit against total # process hits).
Curriculum Vitae

Name: Katherine N. Lee

Post-secondary Education and Degrees:
University of Western Ontario
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2015 – Ontario Graduate Scholarship (OGS)

2014 – CIHR Travel Award, Institute of Circulatory and Respiratory Health (ICRH)

2014 – Clinical and Basic Pharmacology category, UWO Department of Physiology and Pharmacology Research Day, 2nd place

2013 – Platform Presentation award, Great Lakes GPCR Retreat, Cleveland OH

2013 – 3 Minute Thesis Competition finals, UWO, honourable mention

2013 – Graduate Thesis Research Award, Schulich School of Medicine and Dentistry, UWO
**Related Work**

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- 2014-2015, Molecular Techniques

**Publications:**


