Identification and Functional Characterization of Novel Proteins

Regulating Wnt/β-catenin Signaling

A Dissertation Presented

by

Michael Eric Feigin

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Doctor of Philosophy

in

Molecular and Cellular Pharmacology

Stony Brook University

December 2007
Stony Brook University
The Graduate School

Michael Eric Feigin

We, the dissertation committee for the above candidate for the Doctor of Philosophy degree, hereby recommend acceptance of this dissertation.

Craig C. Malbon, Ph.D. - Dissertation Advisor
Leading Professor
Department of Pharmacological Sciences

Jeffrey E. Pessin, Ph.D. - Chairman of Defense
William and Jane Knapp Professor
Department of Pharmacological Sciences

W. Todd Miller, Ph.D.
Professor
Department of Physiology and Biophysics

Howard C. Crawford, Ph.D.
Assistant Professor
Department of Pharmacological Sciences

Howard I. Sirotkin, Ph.D.
Assistant Professor
Department of Neurobiology and Behavior

This dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School
Abstract of the Dissertation

Identification and Functional Characterization of Novel Proteins

Regulating Wnt/β-catenin Signaling

by

Michael Eric Feigin

Doctor of Philosophy

in

Molecular and Cellular Pharmacology

Stony Brook University

2007

The development and maintenance of multicellular organisms requires specific spatial and temporal signaling cues in order to generate and organize complex cell types and tissues. Messages received from the extracellular microenvironment are detected by cellular receptors, thereby activating various intracellular signal transduction cascades and inducing specific responses, including migration, growth, differentiation and death. Disruption of these signaling pathways through genetic mutation results in abnormal embryonic development and a multitude of adult diseases, including cancer. The Wnt/β-catenin signaling cascade controls several vital steps in animal development, including regulation of the body axis and the formation and maintenance of bone. Although intensively studied for over 20 years, the molecular mechanisms by which the Wnt signal is transduced into a cellular response are not fully understood. In this work I seek to
identify and characterize novel Wnt/β-catenin signaling components in an effort to clarify the complex regulation of this critical signaling pathway.

First, I show that RGS19 controls the Wnt/β-catenin pathway through inactivation of the obligate G-protein, Gαo. RGS19 expression specifically attenuated Wnt-induced cellular responses, including gene transcription and differentiation. Overexpression of a constitutively active mutant of Gαo rescued the inhibition of gene transcription caused by RGS19. Expression of RGS19 had no effect on gene transcription induced by Dishevelled3, a downstream activating Wnt signaling element. Finally, knockdown of RGS19 by siRNA suppressed Wnt/β-catenin signaling, suggesting a complex role for RGS19 in this signaling cascade.

Next, I examined the role of OSTM1 in the Wnt/β-catenin pathway. Mutations in the OSTM1 gene were recently found to be the cause of severe autosomal recessive osteopetrosis in both the mouse and humans. Expression of OSTM1 potentiated Wnt-induced signaling events, whereas knockdown of endogenous OSTM1 attenuated the ability of Wnt to stimulate pathway activation. An OSTM1 mutant protein (detected in humans with osteopetrosis) was also found to inhibit Wnt/β-catenin signaling. Finally, expression of wild-type OSTM1 stimulated, whereas mutant OSTM1 inhibited, the Wnt-dependent association of β-catenin with its obligate transcription factor, Lef1. I propose that mutations in OSTM1 result in osteoclastic Wnt/β-catenin signaling defects, leading to autosomal recessive osteopetrosis.

These studies expand our mechanistic understanding of Wnt/β-catenin signal transduction and may lead to generation of therapeutic interventions to the diseases initiated by disruption of this critical regulatory cascade.
# TABLE OF CONTENTS

List of Abbreviations ............................................................................................. vi
List of Figures ......................................................................................................... vii

CHAPTER I – General Introduction ..................................................................... 1

- Wnt signaling in development and disease ............................................................. 1
- Heterotrimeric G-protein regulation of Wnt signaling ........................................... 4
- Structure and function of RGS proteins ................................................................. 7
- Regulation of bone by osteoblasts and osteoclasts .............................................. 10
- Osteopetrosis ......................................................................................................... 14
- Structure and function of OSTM1 ......................................................................... 15
- Regulation of bone mass by Wnt signaling .......................................................... 16
- Figures ................................................................................................................... 18

CHAPTER II - RGS19 Regulates Wnt/β-catenin Signaling Through Inactivation of Gαo ..................................................................................................................... 24

- Introduction .......................................................................................................... 24
- Materials and methods .......................................................................................... 26
- Results .................................................................................................................... 32
- Discussion .............................................................................................................. 43
- Figures ................................................................................................................... 48

CHAPTER III – OSTM1 Regulates β-catenin/Lef1 Interaction and is Required for Wnt/β-catenin Signaling ......................................................................................... 82

- Introduction .......................................................................................................... 82
- Materials and methods .......................................................................................... 85
- Results .................................................................................................................... 89
- Discussion .............................................................................................................. 95
- Figures ................................................................................................................... 98

CHAPTER IV – Discussion and Conclusions .......................................................... 122

List of References .................................................................................................. 127
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>ARO</td>
<td>Severe Autosomal Recessive Osteopetrosis</td>
</tr>
<tr>
<td>Beta2-AR</td>
<td>Beta2-Adrenergic Receptor</td>
</tr>
<tr>
<td>CKIα</td>
<td>Casein Kinase Iα</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>Dvl</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>F9</td>
<td>mouse F9 teratocarcinoma cells</td>
</tr>
<tr>
<td>FAP</td>
<td>Familiar Adenomatous Polyposis</td>
</tr>
<tr>
<td>Fz</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GAIP</td>
<td>G-alpha Interacting Protein</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase Activating Protein</td>
</tr>
<tr>
<td>GIPC</td>
<td>GAIP Interacting Protein C-terminus</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein Coupled Receptor</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen Synthase Kinase 3β</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin antigen</td>
</tr>
<tr>
<td>IB</td>
<td>immunoblot</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal Kinase</td>
</tr>
<tr>
<td>LRP</td>
<td>Low Density Lipoprotein Receptor Related Protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MAS</td>
<td>McCune-Albright Syndrome</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>MITF</td>
<td>Microphthalmia-associated Transcription Factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>Opg</td>
<td>Osteoprotegrin</td>
</tr>
<tr>
<td>OSTM1</td>
<td>Osteopetrosis Associated Transmembrane Protein 1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>primitive endoderm</td>
</tr>
<tr>
<td>PI-3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulator of G-protein Signaling</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse-transcription polymerase chain reaction</td>
</tr>
<tr>
<td>sFRP-1</td>
<td>Secreted Frizzled-related Protein 1</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tartrate-Resistant Acid Phosphatase</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless/int-1</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

CHAPTER I:

Fig 1.1 - Wnt/β-catenin pathway ...............................................................18
Fig 1.2 - Wnt/JNK/PCP pathway ...............................................................20
Fig 1.3 - Wnt/Ca2+/cGMP pathway ...........................................................22

CHAPTER II:

Fig 2.1 - RGS protein expression in F9 cells .............................................48
Fig 2.2 - RGS protein overexpression screen .............................................52
Fig 2.3 - RGS19 attenuates Wnt3a-induced Lef-Tcf reporter activation ..........56
Fig 2.4 - Constitutively active Gaα rescues inhibition of Wnt3a action by RGS19 ....62
Fig 2.5 - RGS19 overexpression attenuates β-catenin stabilization .................66
Fig 2.6 - RGS19 attenuates Wnt/β-catenin signaling upstream of Dvl .............68
Fig 2.7 - RGS19 attenuates Wnt3a-induced formation of primitive endoderm ......72
Fig 2.8 - Knockdown of RGS19 attenuates Lef-Tcf reporter activation .............76
Fig 2.9 - RGS19 mutants attenuate Wnt3a-induced Lef-Tcf reporter activation ....80

CHAPTER III:

Fig 3.1 - OSTM1 mRNA is expressed in F9 cells .......................................98
Fig 3.2 - OSTM1 potentiates Wnt/β-catenin signaling ...............................100
Fig 3.3 - OSTM1 knockdown attenuates Wnt/β-catenin signaling .................104
Fig 3.4 - OSTM1ΔC mutant attenuates Wnt/β-catenin signaling .................108
Fig 3.5 - OSTM1ΔC attenuates Wnt3a-induced PE formation ....................118
Fig 3.6 - OSTM1 regulates β-catenin/Lef1 interaction ...............................120
ACKNOWLEDGMENTS

I would first like to acknowledge my advisor, Dr. Craig Malbon for his invaluable advice and guidance. His patience and open-mindedness gave me the freedom to explore my diverse scientific interests while staying within the scope of the laboratory. This great experience has taught me how to think like a scientist, from performing simple experiments to writing (and re-writing) manuscripts and delivering effective presentations. I would also like to thank Dr. Hsien-yu Wang for her encouragement and training at the start of my career, ensuring I always used proper controls and providing important insight into data analysis and experiment planning.

I thank my committee, Drs. Jeffrey Pessin, Todd Miller, Howard Sirotkin and Howard Crawford, for their insightful questions, ideas and suggestions during my training. I would especially like to thank Dr. Crawford for his willingness to help solve my most basic (and difficult) problems whenever I asked, most often resulting in new experiments proposed from a fresh perspective.

I thank my classmates, Bennett Caces, Jermel Watkins, Martine Mirrione and Elizabeth Scotto-Lavino for making our years at Stony Brook more enjoyable and for all the great memories we shared.

I thank my John Harvards Friday beer group for providing a great way to end a very stressful week and my wine group for erasing the stress on all the other days.

I would like to thank my family for all their love and support. Most importantly, I thank my wife, Erin, who helped me every step of the way and always asked the most important question: When are you finally going to graduate?
Wnt signaling in development and disease

Communication between cells drives the complex processes of embryogenesis and adult tissue homeostasis. Signals secreted or presented from one cell type to another act in a specific spatial and temporal manner to allow proper growth and development. Determining how these signals are sent, received, transduced and function is therefore an important area of research for those interested in the mechanisms of embryology and cancer.

One such signal that has been studied extensively is the Wnt/wingless family of proteins. First identified as a common site of integration for the mouse mammary tumor virus (Nusse and Varmus, 1982), the gene int-1 was classified as an oncogene (Nusse et al., 1984), then shown to be homologous to the segment polarity gene wingless (Rijsewijk et al., 1987). The wingless mutation in Drosophila was characterized by both adult and embryonic phenotypes; imaginal disc defects leading to occasional loss of a wing, and denticle fusion, respectively (Cabrera et al., 1987). The presence of a signal sequence, as well as the genes’ ability to influence areas far from its sites of expression, led to the hypothesis that wingless/int-1 (Wnt) was a secreted protein. It is now known that the Wnt family of secreted proteins, containing nearly 20 mammalian members, controls a wide range of cellular processes, acting through at least three distinct signal transduction...
pathways (Clevers, 2006). The “canonical” Wnt pathway (known as the Wnt/β-catenin pathway) was the first to be characterized and involves the stabilization of the transcriptional co-activator β-catenin, leading to proliferation and differentiation (Logan and Nusse, 2004) (Fig 1.1). The planar cell polarity pathway directs cytoskeletal rearrangement through activation of the c-Jun N-terminal kinase (JNK) cascade (Boutros et al., 1998; Strutt, 2003) (Fig 1.2). Finally, the Wnt/Ca\(^{2+}\)/cyclic GMP pathway leads to changes in intracellular Ca\(^{2+}\), cGMP activity and specific gene expression (Kuhl et al., 2000; Slusarski et al., 1997b) (Fig 1.3). All three signal transduction cascades are initiated through activation of a specific member of the Frizzled family of seven transmembrane G-protein coupled receptors (GPCR) (Bhanot et al., 1996; Malbon, 2004).

The central player in the canonical Wnt pathway is β-catenin, a multifunctional transcriptional co-activator first implicated in cell adhesion (Noordermeer et al., 1994; Siegfried et al., 1994). Under basal conditions, cytosolic levels of β-catenin are tightly regulated through proteasomal degradation and target genes are repressed by members of the Lef/Tcf family of transcription factors (Aberle et al., 1997). Targeting of β-catenin to the proteasome involves phosphorylation on specific N-terminal serine and threonine residues of β-catenin, creating a docking site for the F Box/WD repeat protein β-Trcp and allowing recognition by a ubiquitin ligase enzyme (Kitagawa et al., 1999).Mutations at these residues, which prevent phosphorylation, have been found in several cancers (Polakis, 1999). The protein components necessary for β-catenin phosphorylation, including the scaffold proteins Axin (Zeng et al., 1997) and the adenomatous polyposis coli gene product (APC) (Rubinfeld et al., 1993), and the kinases glycogen synthase
kinase 3β (GSK3β) (Siegfried et al., 1992) and casein kinase 1α (CK1α) (Peters et al., 1999) function in a large degradation complex. β-catenin is recruited to this complex where it is phosphorylated by CK1α and GSK3β (Liu et al., 2002), and subsequently recognized by the ubiquitin system and degraded.

Wnt ligands bind to the co-receptor complex of Frizzled and low-density lipoprotein receptor-related protein (LRP5/6) (Tamai et al., 2000; Wehrli et al., 2000). How these receptors transduce the signal to the interior of the cell is not fully understood. One of the first intracellular changes is the phosphorylation of the Dishevelled (Dvl) protein (Klingensmith et al., 1994). Phosphorylated Dvl is able to disrupt the degradation complex, preventing β-catenin phosphorylation and subsequent degradation. Free β-catenin binds to Lef/Tcf transcription factors and influences the expression of genes important for proliferation and differentiation, including CyclinD1 (Issack and Ziff, 1998) and c-Myc (He et al., 1998).

Wnt signaling controls numerous aspects of embryonic development and is also required for homeostasis of adult tissues (Clevers, 2006). One of the earliest processes in the development of multicellular organisms, formation of the primary axis, requires Wnt3 (Liu et al., 1999) in mice. Genetic knockout analysis of Wnt signaling components has also implicated this pathway in the regulation of limb bud formation (Galceran et al., 1999), hair patterning (Guo et al., 2004) and kidney development (Majumdar et al., 2003), to name but a few examples. Wnt signaling is also required for maintenance of self-renewing tissues in the adult, including the gut (Korinek et al., 1998), hair follicle (van Genderen et al., 1994) and bone (Kato et al., 2002). Mutations in Wnt pathway genes can lead to pathological disturbances in these tissues, as discussed below. Several
human genetic disorders are caused by Wnt pathway mutations reflecting a critical role for Wnt signaling in normal embryonic development (Logan and Nusse, 2004). Mutations in Wnt3 have been shown to cause Tetra-amelia, characterized by loss of limbs (Niemann et al., 2004). Bone density defects, as well as retinal vasculature malformations have been observed in patients with mutations in LRP5 (Boydgen et al., 2002). Finally, dental abnormalities have been reported in humans with Axin2 deficiency (Lammi et al., 2004).

Activating mutations in Wnt/β-catenin pathway components leads to cancer (Lustig and Behrens, 2003). This phenomenon is clearly demonstrated by the hereditary disorder Familial Adenomatous Polyposis (FAP), in which patients inherit one mutant APC allele and develop numerous colon polyps (Kinzler et al., 1991; Kinzler and Vogelstein, 1996). Loss of heterozygosity of APC in these patients causes colorectal cancer (Kinzler and Vogelstein, 1996). Mutations in other Wnt pathway components, including Axin2 (Liu et al., 2000) and β-catenin (Morin et al., 1997), also have been shown to promote colon cancer in humans, confirming the importance of this signaling cascade in normal gut homeostasis. In addition, Axin mutations have been discovered in hepatocellular carcinomas (Satoh et al., 2000), β-catenin mutations in spontaneous pilomatrixomas (Chan et al., 1999) and hepatoblastomas (de La Coste et al., 1998), and APC mutations in prostate cancers (Gerstein et al., 2002).

Heterotrimeric G-protein regulation of Wnt signaling

Recent evidence has shown that the Frizzled receptor belongs to the large class of G-protein coupled receptors (Malbon, 2004), and that heterotrimeric G-proteins are
essential to the Wnt/β-catenin pathway, from Drosophila to vertebrates (Katanaev et al., 2005; Liu et al., 2001; Liu et al., 2005; Malbon, 2004; Malbon, 2005; Slusarski et al., 1997a). Overexpression and suppression studies in mouse F9 teratocarcinoma embryonic stem cells (Liu et al., 2001) as well as flies (Katanaev et al., 2005) have demonstrated that Gαo, for example, is critical for Wnt-stimulated target gene activation.

The first indication that heterotrimeric G-proteins were necessary for Wnt/β-catenin signaling came through the investigation of a chimeric receptor containing the extracellular and transmembrane segments of the β2-adrenergic receptor (β2AR) and the cytosolic domains of Frizzled-1 (Liu et al., 2001). Stimulation of the chimeric receptor by isoproterenol (a β2AR agonist) in F9 cells induced β-catenin accumulation, Lef/Tcf-mediated gene transcription, and primitive endoderm formation, all hallmarks of Wnt/β-catenin pathway activation. Disruption of specific G-protein α-subunits, through pertussis toxin inactivation or antisense oligonucleotide-mediated silencing (Gαo, Gαq), blocked the ability of isoproterenol to induce gene transcription. Conversely, constitutively activated forms of either Gαo or Gαq were able to stimulate Lef/Tcf-mediated gene transcription.

Subsequently, an obligate role for Gαo in the Wnt/β-catenin pathway has been reported in Drosophila (Katanaev et al., 2005). Gαo mutant clones in the wing displayed downregulation of Wnt target genes, while overexpression of Gαo induced increased expression. Importantly, epistasis experiments placed Gαo activity downstream of Frizzled, but upstream of Dishevelled. Finally, embryonic overexpression of Gαo induced a loss of denticles, similar to the phenotype produced upon activation of known Wnt pathway components. Further demonstrating a role for Gαo in Wnt/β-catenin
signaling, G\(\alpha\)o can be coimmunoprecipitated with Frizzled and Dishevelled in a Wnt-dependent manner (Liu et al., 2005).

Heterotrimeric G-proteins are a class of GTP-activated molecular switches composed of a G\(\alpha\)-subunit in complex with a G\(\beta/\gamma\) dimer (Cabrera-Vera et al., 2003). In the inactive state, GDP-bound G\(\alpha\) subunits are complexed with G\(\beta/\gamma\), inhibiting activation of downstream signaling molecules. Upon activation of the G-protein by its cognate heptihelical receptor (i.e., a G-protein coupled receptor, GPCR, such as Frizzled), GDP is exchanged for GTP by the G\(\alpha\) subunit, allowing dissociation of the G\(\beta/\gamma\) dimer. Both the GTP-bound G\(\alpha\)-subunits and the “free” G\(\beta/\gamma\) dimers are available to activate downstream signaling components (Clapham and Neer, 1993), and both have been shown to function in Wnt signaling (Liu et al., 2001; Slusarski et al., 1997a). G-proteins signal to downstream effectors such as ion channels and enzymes involved in second messenger production and lipid modification. For example, activation of G\(\alpha_s\) stimulates the generation of cyclic AMP from ATP by adenylyl cyclase. The period of the activation cycle is determined by the hydrolysis of GTP by the G\(\alpha\) subunit.

Twenty G-protein \(\alpha\)-subunits, five \(\beta\)-subunits and twelve \(\gamma\)-subunits are present in mammals, and homologs have been identified in all developmentally relevant model systems (Malbon, 2005). This has enabled the study of the specific roles of G-protein subunits in development and disease. The first indication that G-proteins mediate multicellular development came in studies of aggregation of the slime mold Dictyostelium discoideum, which requires G-protein-mediated cAMP signaling (Pupillo et al., 1988). Further experiments demonstrated G-protein necessity in neuronal growth
cone regulation (Strittmatter et al., 1990) in rats, gastrulation in *Drosophila* (Parks and Wieschaus, 1991), and adipogenesis in mice (Wang et al., 1992).

Several monogenic human diseases have been associated with loss-of-function or gain-of-function mutations in G-protein alpha subunits (Weinstein et al., 2006). Activating mutations in Goαs (which inhibit GTPase function) are frequently found in pituitary adenomas which secrete growth hormone, and less often in thyroid and adrenal tumors. These mutations also lead to the development of McCune-Albright syndrome (MAS), characterized by fibrous dysplasia of bone, skin hyperpigmentation, sexual precocity and several tumors (Spiegel, 2000). The varied phenotypic outcomes of gain-of-function Goαs mutations are primarily caused by increased cAMP concentrations, leading to abnormal hormone secretion in endocrine tissues, increased proliferation of bone marrow, and altered melanin expression. Inactivating mutations in Goαs result in Albright hereditary osteodystrophy (Majumdar et al.), typified by short stature, mental defects, and obesity, although the exact mechanism for these effects has yet to be determined (Weinstein et al., 2001). In addition, loss-of-function mutations in Goα1 and Goα2 have been associated with stationary night blindness and achromatopsia (congenital color blindness), respectively (Dryja et al., 1996; Kohl et al., 2002).

**Structure and function of RGS proteins**

Heterotrimeric Goα-subunits are known to possess a slow, intrinsic GTPase activity which can be accelerated by a relatively newly discovered class of proteins termed Regulators of G-protein Signaling (RGS proteins) (Hollinger and Hepler, 2002). Over 30 mammalian RGS proteins have been described, each having their own distinct
repertoire of Gα-subunits that they regulate (Hollinger and Hepler, 2002). RGS proteins have been classified into subfamilies (RZ, R4, R7, R12, RA) based upon sequence conservation within the 120 amino acid catalytic RGS domain (Ross and Wilkie, 2000). Members of each subfamily share functional characteristics, including regulation of similar subsets of Gα-subunits. RGS proteins also display modular domains important for protein interactions, for post-translational modifications, and for proper intracellular localization (Burchett, 2000). Several RGS proteins contain cysteine string regions necessary for palmitoylation and subsequent binding to the plasma membrane. Other RGS proteins possess PDZ, PTB and DEP domains necessary for protein complex formation (Burchett, 2000). RGS proteins have been shown to be essential in many G-protein-mediated physiological processes, including visual (Chen et al., 2000) and dopaminergic signal transduction (Rahman et al., 2003), as well as orienting the mitotic spindle during cell division (Malbon, 2005; Martin-McCaffrey et al., 2004). These proteins can act as scaffolds, organizing receptors, G-proteins and their effectors, enhancing efficiency and specificity of signaling interactions (Abramow-Newerly et al., 2006).

The existence of GTPase-activating proteins (GAPs) for heterotrimeric G-proteins was predicted after discrepancies arose between measured rates of purified G-protein GTP hydrolysis (~0.2 Pi/mol Gα/min) and observed rates of deactivation in cellular systems (~200 fold faster) (Gilman, 1987), most prominently in the phototransduction signaling cascade (Arshavsky and Pugh, 1998). Studies in several model organisms were critical in the discovery of RGS proteins and the elucidation of their biological functions. First, a yeast mutant (Sst2p) was uncovered that showed supersensitivity to pheromone (a
G-protein-mediated response) (Chan and Otte, 1982). Next, the *flbA* gene was determined to negatively regulate G-protein signaling in the fungus *Aspergillus nidulans* (Lee and Adams, 1994). In mammalian systems, a novel gene (*GOS8*) was found to be highly expressed in activated monocytes (Siderovski et al., 1994) and G-α-interacting protein (GAIP) was discovered in a yeast two-hybrid screen for Gαi3-binding proteins (De Vries et al., 1995). Finally, the recognition that each of these genes belonged to a conserved class of G-protein-mediating signaling elements was made through sequence analysis of 15 mammalian genes from a brain cDNA library (Koelle and Horvitz, 1996).

A number of mechanisms for RGS-mediated negative regulation of G-protein signaling were possible. Among these were the hypotheses that RGS proteins could act by blocking GTP binding to the Gα-subunit, by decreasing the amount of time the G-protein stayed in the active state, or by inhibiting the interaction of G-proteins with effectors. Several studies quickly determined that RGS proteins act as GAPs for heterotrimeric G-proteins, binding to the transition state of the Gα-subunit for GTP hydrolysis and stimulating a 1000-fold increase in enzymatic activity, thereby decreasing the time spent in the active state (Berman et al., 1996; Hunt et al., 1996; Watson et al., 1996). In addition, RGS proteins can negatively regulate G-protein signaling by binding to Gα-GTP and blocking Gα-effector interactions (Carman et al., 1999; Hepler et al., 1997).

Recent evidence has implicated RGS proteins as critical regulators of development, adult physiology, behavior and disease. The *Drosophila* protein Loco was among the first RGS family members discovered to play a role in early development. Flies lacking Loco fail to hatch, displaying defects in axon sheathing and lack of a blood-
brain barrier (Granderath et al., 1999). Mice genetically engineered to be deficient in RGS14 are also embryonic lethal, due to their inability to complete the first mitosis (Martin-McCaffrey et al., 2004). Several other RGS proteins, including RGS3 and RGS4, can induce migration, proliferation or apoptosis of specific cell types (Dulin et al., 2000; Tatenhorst et al., 2004). Several RGS proteins have been shown to influence heart function, specifically through regulation of $K^+$ channels driving cardiac excitability (Fujita et al., 2000). Immune function can also be controlled by RGS proteins, as RGS2 knockout mice display deficits in T-cell activation (Oliveira-Dos-Santos et al., 2000).

Among the most studied aspects of RGS protein biology is the role of this class of proteins in neuronal function and behavior. In *C. elegans*, Egl-10 regulates egg laying and the food deprivation response (Dong et al., 2000). RGS2 knockout mice display increased anxiety and hippocampal defects have been reported (Oliveira-Dos-Santos et al., 2000). Finally, RGS9 knockout mice (and humans with similar genetic defects) are unable to respond to changes in light intensity due to slow deactivation of the phototransduction cascade (Nishiguchi et al., 2004). Several RGS proteins have also been implicated in disease states, including RGS16 in retinitis pigmentosa (Bressant et al., 2000), RGS8 in prostate cancer (Sood et al., 2001), RGS4 in schizophrenia (Mirnics et al., 2001) and RGS9 in Parkinson’s disease (Tekumalla et al., 2001).

**Regulation of bone by osteoblasts and osteoclasts**

The maintenance of bone is a critical process involving two distinct cell types: osteoblasts (which build bone) and osteoclasts (which break down bone). Defects in bone resorption or formation lead to diseases such as osteoporosis, a pathological loss of
bone mass (Teitelbaum and Ross, 2003). Osteoblasts are generated from mesenchymal stem cells and are responsible for secreting osteoid, a combination of bone matrix proteins, including type I collagen, onto bone surfaces (Mackie, 2003). The collagen provides a structure for further deposition of bone material. In addition to collagen, osteoblasts produce specific proteoglycans and glycoproteins which regulate several processes including collagen fibrillogenesis, and bone cell adhesion and differentiation. Osteoblasts are also responsible for bone mineralization, in which crystals of hydroxyapatite are nucleated by phosphate-containing bone sialoprotein. Finally, osteoblasts regulate bone mineralization though production of alkaline phosphatase, an enzyme that controls the phosphorylation state of several critical bone matrix proteins. After completing the process of localized bone deposition, osteoblasts either undergo apoptosis or become surrounded by bone matrix and form osteocytes. Several human diseases result from defects in osteoblast function, including osteoporosis, Paget’s disease and osteoarthritis.

Osteoclasts are generated from the fusion of haemopoietetic precursors, resulting in large, polarized, multinucleated cells of the monocyte/macrophage family (Teitelbaum and Ross, 2003). A hallmark of osteoclasts is the ruffled membrane, the resorptive organelle containing vacuolar proton pumps (H^+ ATPases) necessary for bone matrix degradation (Blair et al., 1989). This specialized membrane structure is only present when an osteoclast comes in contact with the skeletal surface. The first step in osteoclast formation is mediated by PU.1, an ETS domain transcription factor. Mice deficient in PU.1 lack bone marrow macrophages, and therefore, do not form osteoclasts (Tondravi et al., 1997). Interaction of PU.1 with members of the microphthalmia-associated
transcription factor (MITF) family of proteins allows late monocytic cells to reach an early osteoclast precursor state. However, osteoclasts lacking the microphthalmia locus are unable to multinucleate or form ruffled membranes (Thesingh and Scherft, 1985), due to the inability to induce transcription of specific target genes, including tartrate-resistant acid phosphatase (TRAP) and carbonic anhydrase II (Luchin et al., 2000; Luchin et al., 2001). In addition, multinucleated osteoclasts lacking the microphthalmia locus rapidly undergo apoptosis, as the anti-apoptotic gene Bcl-2 is an MITF target gene. Monocytic precursors are further pushed to an osteoclastic fate by several factors produced by osteoblasts, including macrophage colony-stimulating factor (M-CSF) and RANKL (Yao et al., 2002). M-CSF induces numerous osteoclastic events, including proliferation, cell spreading, motility and cytoskeletal reorganization (Insogna et al., 1997). These effects are mediated through activation of c-Src and phosphatidylinositol-3 kinase (PI-3K), downstream effectors of c-Fms, the M-CSF receptor (Grey et al., 2000).

Full differentiation of an osteoclastic precursor into a committed osteoclast requires contact with osteoblasts (Udagawa et al., 1990). A member of the tumor necrosis factor protein family, RANKL, expressed on the surface of osteoblasts and their precursors, binds to the RANK receptor, expressed on osteoclasts (Lacey et al., 1998; Yasuda et al., 1998). Interaction of RANK and RANKL induces mitogen activated protein kinase (MAPK) cascades, as well as NFκB and PI-3K, culminating in osteoclast differentiation and cytoskeletal rearrangement. Mutations in several of the genes discussed above lead to either osteoclast-deficient or osteoclast-dysfunctional osteopetrosis, a disorder of high bone mass. Interestingly, almost all osteopetrosis patients with defined genetic defects belong to the osteoclast-dysfunctional family.
Interaction between the committed osteoclast and the bone matrix is required for proper bone degradation, and several molecules are obligatory for this attachment. Among these, the osteoclast-embedded αvβ3 integrin binds to bone matrix proteins and induces podosome formation (McHugh et al., 2000). These podosomes define the sealing zone, isolating the region of bone resorption. Additionally, the integrins act at the bone surface to polarize the osteoclast, allowing directed trafficking of acidified vesicles containing the H⁺ ATPase and generating the ruffled membrane (Abu-Amer et al., 1997). This cell polarization event requires the small GTPases Rho and Rac (Faccio et al., 2003).

Bone degradation begins with acidification of the local microenvironment between the bone surface and the bound osteoclast, thereby exposing the organic matrix. Osteoclastic carbonic anhydrase II generates protons, which are then moved through the H⁺ ATPase, lowering the pH in the bone degradation zone to ~4.5 (Sly et al., 1983). The excess bicarbonate molecules produced by the carbonic anhydrase enzyme are exchanged for chloride ions in the non-resorptive membrane. Finally, the chloride ions enter the resorptive zone through a charge-coupled anion channel (Schlesinger et al., 1997). This exchange is required to maintain osteoclastic electron balance. Importantly, humans lacking functional Clc-7, the main resorptive chloride channel, present with osteopetrosis (Kornak et al., 2001). The organic material in the bone is degraded by cathepsin K, a collagenolytic enzyme which acts at low pH levels and is released by exocytosis of osteoclastic vesicles (Gelb et al., 1996). Osteoclastic action results in a ~50μm deep resorption pit that is then filled with new bone material by osteoblasts. Healthy skeletal
systems maintain proper bone homeostasis through equivalent levels of bone resorption and formation.

**Osteopetrosis**

Defects in osteoclastic resorptive function cause osteopetrosis, a rare human genetic disorder (Del Fattore et al., 2007). Failure of the body to effectively break down bone leads to hematological defects due to lack of bone marrow space, and sight and hearing loss caused by closure of bone foramina around the cranial nerves. Additionally, osteopetrosis is associated with osteosclerosis, shortness of stature and bone fragility. Although the disease is generally fatal at an early age, bone marrow transplant can be curative in 50% of patients (Driessen et al., 2003). However, this treatment is unable to reverse the short stature and visual impairment defects. Mutations in several human genes have been shown to cause osteopetrosis of differing severity, all resulting in defective osteoclast function. Humans lacking a functional *TCIRG1* gene, encoding the α3 subunit of the H⁺ ATPase in the resorptive membrane, present with severe osteopetrosis and account for over 50% of cases. Mutations in the *ClCN7* and *OSTM1* genes generate similar severe phenotypes, in addition to neurodegeneration (Kasper et al., 2005; Kornak et al., 2001; Lange et al., 2006). *ClCN7* encodes the Clc-7 protein, the main resorptive chloride channel. The function of the *OSTM1* protein and its role in osteopetrosis will be discussed in greater detail.
Structure and function of OSTM1

The spontaneous grey-lethal mouse has been used as a model for over 70 years to study the molecular and cellular defects underlying severe autosomal recessive osteopetrosis (Rajapurohitam et al., 2001). These mutant mice display a grey coat color due to defects in melanocyte maturation, lack of tooth eruption, decreased medullary space and increased bone density. On the cellular level, osteoclasts isolated from grey-lethal mice are unable to develop a ruffled border and show impaired resorptive function (Rajapurohitam et al., 2001). Positional cloning and genetic complementation in transgenic mice allowed identification of OSTM1 as the causative mutation for the osteopetrotic phenotype (Chalhoub et al., 2003). The OSTM1 gene encodes a 338 amino acid type I transmembrane protein with homologs in several multicellular organisms. However, no similar genes within a specific species have been identified through sequence database searches. Several human mutations in the OSTM1 gene have been identified, each leading to the production of a C-terminally truncated protein, lacking the transmembrane domain. Immunofluorescence microscopy studies have determined that OSTM1 localizes to late endosomes and lysosomes, as well as the plasma membrane (Lange et al., 2006), with the short C-terminal region facing the cytosol. The larger N-terminal segment is post-translationally cleaved, with the two segments remaining bound through disulfide bond interactions. Additionally, several amino acids in the N-terminal domain may be glycosylated (Lange et al., 2006). It has been reported that OSTM1 is required for stabilization and proper localization of Clc-7 (Lange et al., 2006). Independently, OSTM1 was identified in a yeast two-hybrid screen for proteins interacting with RGS19 (Fischer et al., 2003). Overexpression analysis of OSTM1
suggested that the protein acts as an E3 ubiquitin ligase for the heterotrimeric G-protein, Gαi3.

**Regulation of bone mass by Wnt signaling**

The realization that canonical Wnt/β-catenin signaling regulates bone development and maintenance was made upon the discovery of human mutations in LRP5 that generated specific skeletal defects (Krishnan et al., 2006). Loss-of-function mutations in LRP5 were shown to be present in patients suffering from osteoporosis-pseudoglioma syndrome, a disorder of low bone mass (Gong et al., 2001). Alternatively, activating mutations in LRP5 caused a high bone mass phenotype (Ai et al., 2005; Boyden et al., 2002). Subsequent studies in genetically engineered mice have revealed that Lrp5<sup>-/-</sup> animals have decreased numbers of osteoblasts underlying the bone formation defect, while osteoclast function appears unaffected (Kato et al., 2002).

Since Wnt ligands can activate several distinct signaling pathways, the molecular mechanism by which defects in LRP5 led to altered bone development were investigated further. Through the examination of numerous conditional knockout mice, it became apparent that the Wnt/β-catenin pathway was responsible for several steps in the development and maintenance of bone. For example, inactivation of β-catenin in chondrocytes (a cell type that is generated from an osteoblast progenitor) led to increased chondrocyte proliferation and decreased osteoblast differentiation (Day et al., 2005; Hill et al., 2005). β-catenin also plays an intrinsic role in osteoblast regulation of osteoclast differentiation. Mice in which β-catenin is activated specifically in osteoblasts present
with high bone mass due to increased *osteoprotegrin* (*Opg*) expression, a potent inhibitor of osteoclast differentiation (Glass et al., 2005).

Most models suggest that activated Wnt/β-catenin signaling leads to a high bone mass phenotype, nearly always affecting osteoblastic progenitor proliferation and differentiation. However, Wnt signaling may play a different role in the osteoclast. Activation of the Wnt/β-catenin pathway by inhibition of secreted Frizzled-related protein-1 (sFRP-1, a Wnt signaling antagonist), potentiated osteoclast differentiation and function (Hausler et al., 2004). This finding supports the data from sFRP null mice, which display increased osteoclast formation in ex vivo assays (Bodine et al., 2004). Therefore, it is apparent that Wnt/β-catenin signaling plays complex roles in bone development, with pathway activation generating a multitude of developmental effects in specific spatial and temporal patterns.
Figure 1.1 – A schematic of the canonical Wnt/β-catenin pathway.
Fig 1.1

Frizzled 1

Gα

Dvl

APC

β-cat

GSK-3β

Axin

Proteasomal degradation

β-cat

β-cat

β-cat

β-cat

Lef/Tcf

Myc, cyclinD1, Lefty1
Figure 1.2 – A schematic of the Wnt/Ca$^{2+}$ pathway.
Fig 1.2

Frizzled 2

Wnt5A

Wnt5a

PDEα

PDEβ

cGMP

GMP

Ca^{2+}

Calcineurin

IP3

DAG

Ca^{2+}

PKC

TFs

NF-AT

REST, Groucho
Figure 1.3 – A schematic of the PCP pathway.
**Fig 1.3**

- **Wnt3a**
- **Frizzled 1**
- **G_α_**
- **Dvl**
- **RhoA, RhoB, Rac1, Cdc42**
- **MEKK1,4**
- **MKK4, MKK7**
- **JNK**
- **c-Jun phosphorylation**
- **c-Jun dependent transcription**
- **Planar cell polarity**
CHAPTER II

RGS19 Regulates Wnt/β-catenin Signaling Through Inactivation of Gαo

Introduction

In unstimulated cells, cytosolic levels of the transcriptional co-activator β-catenin are tightly regulated by a multi-protein complex that includes GSK3β, Axin, and APC (Ikeda et al., 1998; Kishida et al., 1998; Zeng et al., 1997). Within the complex, β-catenin is phosphorylated and thereby targeted for degradation by the proteasome (Aberle et al., 1997). Upon binding of Wnt3a to its cognate cell surface heptihelical Frizzled (Fz1), the degradation complex is inhibited, allowing β-catenin to accumulate in the cytosol, translocate to the nucleus, and activate target genes such as c-myc and cyclinD1, via Lef-Tcf-sensitive transcription (Molenaar et al., 1996). The mechanism by which stimulation of Frizzled leads to the inhibition of the degradation complex for β-catenin is not fully understood, although it does involve activation of the phosphoprotein Dishevelled (Dvl) (Yanagawa et al., 1995).

Heterotrimeric G-proteins are essential to the Wnt/β-catenin pathway, from Drosophila to vertebrates (Katanaev et al., 2005; Liu et al., 2001; Liu et al., 2005; Malbon, 2004; Malbon, 2005; Slusarski et al., 1997a). Gain- and loss-of-function studies in mouse F9 teratocarcinoma embryonic stem cells (Liu et al., 2001) as well as flies (Katanaev et al., 2005) have demonstrated that Gαo, for example, is critical for Wnt-
stimulated target gene activation. Heterotrimeric Go-subunits are known to possess a slow, intrinsic GTPase activity which can be accelerated by a class of proteins termed Regulators of G-protein Signaling (RGS proteins) (Hollinger and Hepler, 2002).

As Wnt signaling is under tight regulatory control that requires heterotrimeric G-proteins, we hypothesized that RGS proteins themselves may play a role in modulating output of Wnt signaling, and specifically, our first analysis was of the Wnt/β-catenin pathway. We show that RGS19 specifically controls signaling of the Wnt/β-catenin pathway. Expression of RGS19 attenuates Dvl phosphorylation, β-catenin accumulation, Wnt-responsive gene transcription, and blocks Wnt-induced differentiation of mouse F9 teratocarcinoma cells via inactivation of Goα. Knockdown of RGS19 protein expression also suppresses Wnt-induced gene transcription and β-catenin accumulation, suggesting a complex role for RGS19 in the regulation of Wnt/β-catenin signal transduction.
Materials and Methods

Plasmids

Mammalian expression vector pcDNA3.1 encoding RGS3, 5, 9, 10, 14, 16, 17, 19, and 20, Q205L GαoA, Q209L Gα11 and Q209L Gαq were obtained from UMR cDNA Resource Center (University of Missouri-Rolla, MO). Expression vectors containing Fz1 and M50 were provided by Dr. Randall Moon (Department of Pharmacology, HHMI, University of Washington, Seattle, WA). Expression vectors encoding RGS11 and RGS12 were provided by Dr. David Siderovski (University of North Carolina, Chapel Hill, NC).

Antibodies

The rabbit antibody against RGS19 was provided by Dr. Susanne Mumby (Department of Pharmacology, University of Texas, Southwestern Medical Center, Dallas, TX). The monoclonal TROMA-1 antibody, recognizing the primitive endoderm marker cytokeratin endo A, was generated by the University of Iowa Developmental Studies Hybridoma Bank (Iowa City, IA). The rabbit antibody against β-catenin and the mouse antibody against actin were obtained from Sigma (Saint Louis, MO). The mouse antibody against Dishevelled3 (4D3) and the antibodies against Gαo and Gα11 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The rat antibody against the hemagglutinin antigen (HA) was obtained from Roche (Indianapolis, IN). The antibody recognizing Gaq was obtained from BD Biosciences (San Jose, CA)
Cell culture and transfection

Mouse F9 teratocarcinoma cells were obtained from the ATCC (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum plus penicillin (60 µg/ml) and streptomycin (100 µg/ml) in a humidified atmosphere chamber supplied with 5% CO₂ at 37° C. Cells were transfected by using Lipofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer’s suggested protocol.

Reverse-transcription polymerase chain reaction

Specific primers for amplification of regions of each RGS gene and cyclophilin A were obtained from Operon (Huntsville, AL). The sequences are as follows: RGS1: gaaaaactcttgcccaacca, tctgtgtgccagagtcgc; RGS2: gaggagaacggggagaaat, gaggacagtttttggtgga; RGS3: cctggaaagttgtctcttc, tagagtcagacggagaa; RGS4: cctgcgaacacagtttctca, gagacgagggagtgctcag; RGS5: gaagatcaagtcctgcccatca, gcctgtgatttgcctgttt; RGS13: tgacgccagctcttctgg, ttggaatgtcctcctttgg; RGS16: cctgctggagagagctaaa, tgagtgctcgttcgctc; RGS18: catgggtcagggaaaga, cctgctttgctcgtacca; RGS10: gaagcagatgcagggagaa, ggtggaaggtcgctcattg; RGS12: tgcctttctagatggggtg, actgggccacccgctctag; RGS14: tctctgccagctgacct, gggatgctctctgtctgc; RGS20: gagcctcccagctgctgta, gcatggagccgctacctg; Conductin: ctecccaacctgaatgga, agaggtgtcctcagaaat; RGS6: agaaccagacaagcctgaa, ctgattgctgctgttgtg; RGS7: ttatgaccagaggcaagc, cagcttgctgctgctgct; RGS9:
ccgatttcagcgcatt, tgggtctgtctttatcca; RGS11: ccatcatgtcagggtgtctg, aggagaacacaacccacag; CycA: agcactggagaaaggttg, cacaatgttcatgccttcttc. RNA was isolated from wild-type F9 cells using the RNA STAT-60 reagent (Tel-Test, Friendswood, TX) according to the manufacturer’s suggested protocol. Briefly, medium was removed from confluent cultures of F9 cells, followed by addition of RNA STAT-60. Cells were pipetted vigorously into microfuge tubes and incubated at room temperature for 5 minutes, then vortexed in the presence of chloroform. After a brief centrifugation, the upper aqueous layer was collected into a fresh tube. Three phenol/chloroform extractions were performed, followed by isopropanol precipitation. The RNA pellet was washed with 75% ethanol and air-dried. Finally, the RNA was dissolved in DEPC-treated water and stored at -80° C. The reverse transcription reaction was then performed using the Invitrogen Superscript system. RNA (0.7 µg/µl) was mixed with oligo dT15 primers and incubated for 3 minutes at 85° C. First strand buffer, DTT and dNTPs were added and incubated at 42° C for 2 minutes. Finally, Superscript II reverse transcriptase was added and incubated at 42° C for 1 hour. The reaction was stopped by heating the mixture to 85° C for 10 minutes.

Primer sets (0.5 µM) were mixed with dNTPs (1 mM), F9 cDNA (100 ng), 10X Pfu buffer and Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). The PCR reaction was performed using the GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA) with the following protocol: 95° C, 5 minutes; (95° C, 1 minute; 60° C, 1 minute; 72° C, 3 minutes) 30 cycles; 72° C, 15 minutes. Reaction products were resolved electrophoretically upon 1% agarose gels and then stained and made visible with ethidium bromide.
**Membrane preparation**

Confluent F9 cells were washed with ice-cold phosphate-buffered saline (PBS), detached from the culture plate with PBS containing 2 mM EDTA and collected by centrifugation. The cells were resuspended in buffer composed of 20 mM Hepes, pH 7.4, 2 mM MgCl₂, and 1 mM EDTA (HME buffer) plus leupeptin (5 µg/ml), aprotinin (5 µg/ml) and PMSF (200 nM), and incubated on ice for 15 minutes. Homogenization was performed with a Dounce homogenizer and nuclei were removed by a low speed centrifugation (2000 rpm). The post-nuclear supernatant was then centrifuged at 10,000 rpm and the “crude membrane” pellet resuspended in HME buffer. The Lowry method was used to determine protein concentration.

**Gene transcription assay**

F9 cells were seeded into 12-well plates and transiently transfected as described above. Following incubation for 48 hours at 37°C, cells were serum-starved overnight, then treated with purified Wnt3a (R&D Systems, Minneapolis, MN). Cells were lysed directly on the plates through addition of diluted Cell Culture Lysis Reagent 5X (Promega, Madison, WI). Lysates were collected into chilled microfuge tubes on ice and centrifuged at 15,000 x g for 5 minutes. The supernatant was transferred into a new tube and directly assayed as described below. A sample (20 µl) of lysate was added to 100 µl of luciferase assay buffer (20 mM Tricine, pH 7.8, 1.07 mM MgCO₃, 4 mM MgSO₄, 0.1 mM EDTA, 0.27 mM coenzyme A, 0.67 mM luciferin, 33.3 mM DTT, ATP) and a luminometer (Berthold Lumat LB 9507) was used to measure luciferase activity.
**Cytosolic \( \beta \)-catenin assay**

In order to separate cytosolic from membrane-associated \( \beta \)-catenin, samples were treated first with concanavalin A (Con A) (Aghib and McCrea, 1995). Con A (covalently linked to Sepharose) was obtained from Amersham Biosciences (Upsala, Sweden). Confluent F9 cells were washed once with PBS and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) plus leupeptin, aprotinin and PMSF. After collection into microfuge tubes on ice, lysates were rotated at 4° C for 20 minutes and then centrifuged for 25 minutes at 15,000 rpm. The protein concentration of the supernatant fraction was determined and lysates were diluted with RIPA buffer to a protein content of 2.5 mg/ml. Sixty \( \mu \)l of Con A-Sepharose was added to each sample which then was incubated at 4° C for 1 hour with rotation. After a brief centrifugation the supernatant was aspirated into a new tube, 30 \( \mu \)l of Con A-Sepharose was added and the samples were rotated for another hour. Finally, the supernatant was removed and the protein concentration determined. The samples were subjected to SDS-PAGE on 10% acrylamide gels, the separated proteins transferred to nitrocellulose blots, and the blots probed with the anti-\( \beta \)-catenin antibody.

**Indirect immunofluorescence**

F9 cells grown in 24-well plates were fixed in 3% paraformaldehyde, then washed three times in MSM-PIPES buffer (18 mM MgSO\(_4\), 5 mM CaCl\(_2\), 40 mM KCl, 24 mM NaCl, 5 mM PIPES, 0.5% Triton X-100, 0.5% NP40). Cells were incubated at 37° C for 30 minutes with the TROMA antibody, washed with MSM-PIPES, and then incubated at 37° C for 30 minutes with an anti-mouse antibody coupled to Alexa Flour 488.
(Invitrogen). Cells were then washed in blotting buffer (560 mM NaCl, 10 mM KPO4, 0.1% Triton X-100, 0.02% SDS) and imaged with a Zeiss LSM510 inverted fluorescence microscope.

**siRNA vector generation**

The *pSilencer* siRNA expression vector from Ambion (Austin, TX) was used to drive expression of specific siRNAs in F9 cells. A DNA sequence encoding a short hairpin RNA targeted against RGS19 was cloned into the *pSilencer* vector according to the manufacturer’s instructions. Sense and antisense oligonucleotides were generated by Operon (Huntsville, AL). The sequences are as follows: Sense: gatccgccctaaggaagtacagagttcaagagactctgtacttccttagggctta; Antisense: agcttaagccctaaggaagtacagagtctcttgaactctgtacttccttagggcg. The siRNA vectors were transfected into F9 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s suggested protocol. Cell lysates were collected 72h after transfected and RGS19 protein levels determined.
Results

RGS protein expression in F9 cells: analysis at the mRNA level

The expression of RGS family members was investigated in mouse F9 teratocarcinoma (F9) cells. Mouse F9 cells have been used extensively to model early stages of mouse development (Lehtonen et al., 1989). These totipotent stem cells can be induced to differentiate into primitive endoderm by several morphogens, including retinoic acid (Strickland and Mahdavi, 1978) and Wnt3a (Liu et al., 2001). F9 cells also have been used in studies of Wnt/β-catenin signal transduction due to their expression of key mediators of the pathway including Gαo, Gαq, GSK3β, Axin, β-catenin and Dvl (Liu et al., 2001). Reverse transcription-polymerase chain reaction (RT-PCR) was employed to probe the presence of mRNA of RGS proteins in F9 cells. Specific primers were designed for each nucleotide sequence and validated for possible cross-reactivity through homology searches on NCBI-BLAST. RNA was isolated from F9 cells and reverse transcribed to synthesize cDNA. Expression of thirteen RGS proteins was detected (Fig. 2.1A, B), including members of each of the five RGS subfamilies: R4 (RGS3, RGS5, RGS16); R12 (RGS10, RGS12, RGS14); RZ (RGS20, RGS17, RGS19); RA (Axin, Conductin); and R7 (RGS9, RGS11).

RGS protein overexpression screen

The ability of overexpression of those RGS proteins identified in F9 cells to regulate Wnt/β-catenin signaling was examined. Wnt3a stimulation of Fz1 leads to β-catenin accumulation and activation of β-catenin-sensitive, Lef-Tcf-dependent transcription (Molenaar et al., 1996). In order to probe the possible role of RGS proteins
in Wnt/β-catenin/Lef-Tcf signaling, all RGS proteins found in the RT-PCR screen were transiently expressed in the embryonic F9 cells (excluding the known Wnt signaling components Axin (Zeng et al., 1997) and Conductin (Behrens et al., 1998)). The well known promoter target of Wnt3a action, Lef-Tcf-sensitive transcription, was measured using a luciferase-based β-catenin-responsive promoter system. The reporter, termed M50, contains eight consensus Tcf-binding sites upstream of a minimal promoter driving luciferase expression (Veeman et al., 2003). Expression of only RGS19 was found to attenuate Wnt3a-induced gene transcription by more than 50% (Fig. 2.2A), while basal transcriptional levels remained unaltered (data not shown). Overexpression of other RGS proteins, however, including RGS3, 5, 9, 10, 11, 12, 14, 16, 17 and 20, had no effect on Wnt3a stimulation of Lef-Tcf-sensitive transcriptional activation, or on basal transcriptional levels (Fig. 2.2A and data not shown). Western blot analysis of HA-tagged RGS proteins showed similar levels of expression for the various constructs tested (Fig. 2.2B). RGS19 expression had no effect on a control reporter plasmid containing eight mutated Tcf-binding sites (data not shown). In addition, RGS19 was a high-value target as the PDZ domain-containing adaptor protein GIPC (GAIP-interacting protein C-terminus) has been shown to interact with both RGS19 (De Vries et al., 1998) and Frizzled (Tan et al., 2001). Both overexpression and suppression of GIPC inhibits neural crest induction by Frizzled3 in Xenopus (Tan et al., 2001). Based upon these observations, RGS19 was selected for more detailed study of its role in Wnt/β-catenin signaling.

The results from the RT-PCR analysis of RGS19 mRNA was extended to the level of RGS19 protein expression. Crude cytosolic and cell membrane subcellular
fractions were prepared from F9 cells, the proteins separated by SDS-PAGE and subjected to immunoblotting (IB). One band ($M_r = 29$ kDa) in the cytosolic fraction and two bands ($M_r = 27$ and 29 kDa) in the membrane preparation were detected prominently by an RGS19-specific antibody (Fig. 2.2C). The doublet of RGS19 associated with the membrane is likely to reflect the known protein phosphorylation of this RGS protein, as evidenced by the reduction in the upper band after treatment with alkaline phosphatase, and as described in previous reports (Fischer et al., 2000).

**RGS19 overexpression attenuates Wnt3a-induced Lef-Tcf reporter activation**

A time-course for Wnt3a-stimulated gene transcription was performed in F9 cells. Wnt3a stimulated a ~6-fold increase in gene transcription (as measured by luciferase activity, Fig. 2.3A). Wnt-stimulated gene transcription displayed a sharp increase at six to eight hours following stimulation by Wnt3a. Maximal Wnt-stimulated activity was observed at 10 hours, declining thereafter. Wnt/β-catenin signaling was probed in F9 cells transiently transfected with an expression vector harboring RGS19. Expression of RGS19 was found to attenuate the maximal response of Wnt3a-stimulated gene transcription. Transient expression of RGS19 under these conditions suppressed the Wnt3a-induced response by at least 50%. Basal activity and the kinetics of the reporter gene response, in contrast, were largely unaffected by RGS19 expression.

In order to detail the relationship between RGS19 overexpression and Wnt3a-stimulated β-catenin/Lef-Tcf transcription, we sought to establish a dose-response relationship for increased expression of RGS19 protein and the Wnt-stimulated gene transcription response. Increasing levels of RGS19 expression were found to
progressively inhibit Wnt3a-mediated gene transcription (Fig. 2.3B). In the absence of exogenous RGS19, Wnt3a promoted a ~6-fold increase in reporter activation. At the lowest level of RGS19 expression tested (0.05 µg DNA/well), a small, but reproducible decrease in Wnt3a-stimulated transcription also was observed. At higher levels of RGS19 expression (0.2 µg DNA/well), Wnt-stimulated gene transcription was inhibited by 33%. At the highest level of RGS19 expression tested (0.4 µg DNA/well) both Wnt3a-stimulated as well as basal transcriptional activity were found to be markedly suppressed. The expression level of HA-tagged RGS19 agrees well with the relative amount of DNA used in the transfections, as documented by immunoblotting of whole-cell extracts subjected to SDS-PAGE and immunoblotted with an anti-HA antibody (Fig. 2.3B). The expression of HA-RGS19 at the highest level tested represents less than 10% of endogenous RGS19 (data not shown).

To address the specificity of the effect of RGS19 protein overexpression, a homologous member of the RZ subfamily (RGS17) was also expressed to analyze its influence on Wnt3a/β-catenin signaling. RGS17 is 58% identical (76% similar) in amino acid sequence to RGS19. RGS17 has been shown in vitro to accelerate the GTPase activity of several Gα subunits (Mao et al., 2004). Expression of RGS17 was found to have no effect on Wnt3a-induced activation of Lef-Tcf-sensitive transcription (Fig. 2.3C). Increased expression of RGS19, in contrast, progressively inhibited Wnt3a-mediated gene transcription (Fig. 2.3B, C). Expression of RGS17 did not alter Lef-Tcf transcription, even at the highest levels of transfection with the same expression vector harboring RGS19. Expression levels of HA-tagged RGS17 and HA-tagged RGS19 were similar, as documented by immunoblotting of whole-cell lysates subjected to SDS-PAGE
and detected with an anti-HA antibody (Fig. 2.3D). Thus, expression of RGS19 protein attenuates the Wnt3a/β-catenin/Lef-Tcf pathway in a specific, dose-dependent, and time-dependent manner, whereas expression of the highly homologous RGS17 protein does not.

Constitutively active Gαo rescues inhibition of Wnt3a action by RGS19

RGS proteins regulate heterotrimeric G-protein signaling by accelerating GTP hydrolysis by the activated Gα-subunit. If the hypothesis that RGS19 attenuates Wnt3a function by this mechanism is correct, then expression of a mutant G-protein that remains in the active state would be expected to rescue the RGS effect. We tested the ability of the expression of constitutively active Gα-subunits to rescue the inhibitory effects of RGS19 on Wnt3a-mediated gene transcription (Fig. 2.4A). Transient transfection with an expression vector harboring the constitutively active Q205L mutant of Gαo was able to rescue Wnt3a-stimulated Lef-Tcf activation in cells expressing RGS19 protein (Fig. 2.4A, left-handed panel). Expression of the constitutively active Q209L mutant of Gα11, or the constitutively active Q209L mutant of Gαq, in contrast, failed to rescue the Wnt3a-stimulated transcription in RGS19 expressing cells (Fig. 2.4A, right-handed and bottom panels). Expression of either G-protein alone had no effect on Wnt3a-stimulated gene transcription. Expression of each G-protein was established by immunoblotting of whole-cell lysates subjected to SDS-PAGE and detected with antibodies specific for each protein (Fig. 2.4B). Note that the mutant form of Gαo displays a small, but reproducibly greater mobility in these gels.
RGS19 expression attenuates β-catenin stabilization that results from Wnt3a stimulation

A hallmark of canonical Wnt signaling is the intracellular accumulation of β-catenin (Peifer et al., 1994). If our hypothesis that RGS19 expression accelerates deactivation of Gαo and thereby suppresses Wnt3a-stimulated Lef-Tcf-sensitive transcription is correct, then one would predict that overexpression of RGS19 should counter the stabilizing effect of Wnt3a on intracellular β-catenin accumulation (Fig. 2.5). Basal levels of cytosolic β-catenin are low in the absence of stimulation of the canonical pathway by Wnt3a (Fig. 2.5A). Upon stimulation by Wnt3a, β-catenin accumulates in the cytosol, with a peak of intracellular accumulation of β-catenin occurring at 2-3 hours. This Wnt3a stimulated accumulation of β-catenin was observed for more than 5 hours (Fig. 2.5A). We tested our hypothesis by overexpressing RGS19 in F9 cells and then measuring β-catenin levels in the cytosol of cells not treated with Wnt3a (time = 0). β-catenin levels were suppressed in F9 cells overexpressing RGS19 protein (time = 0). The intracellular accumulation of β-catenin in response to stimulation by Wnt3a was obvious in control cells and clearly countered by overexpression of RGS19 protein. The maximal level of β-catenin still occurs at ~3 hours in cells expressing RGS19 protein, but remains well below the β-catenin levels observed in the cytosol of cells transfected with an empty vector. Quantification of the data from multiple experiments confirms, at the level of β-catenin, our hypothesis that RGS19 regulates Wnt3a control of Lef-Tcf transcription (Fig. 2.5B).
**RGS19 attenuates Wnt/β-catenin signaling upstream of Dvl**

Phosphorylation of Dvl3, an initial cellular response to stimulation of the canonical pathway by Wnt3a, is upstream of β-catenin degradation and downstream of activation of Frizzled-1 (Gonzalez-Sancho et al., 2004; Yanagawa et al., 1995). Analysis by SDS-PAGE reveals non-phosphorylated and phosphorylated forms of Dvl3 in F9 cells (Fig. 2.6A). In the absence of Wnt stimulation (*i.e.*, 0 minutes), two Dvl3 isoforms (*M*<sub>r</sub> = 82, 85) are apparent, their presence presumed to reflect different degrees of protein phosphorylation. To establish if these isoforms of Dvl3 are truly differentially phosphorylated forms of the protein we treated the samples with purified calf intestinal alkaline phosphatase (Fig. 2.6B). Upon alkaline phosphatase treatment only the highest mobility band remains, providing evidence to support the tenet that the slower mobility band is indeed the phosphorylated form of Dvl3.

Treating wild-type F9 cells with Wnt3a provokes a sharp increase in the relative amounts of slower migrating phospho-Dvl3 (Fig. 2.6A). In response to Wnt3a, the slowest mobility band (*i.e.*, labeled p-Dvl3 = phospho-Dvl3) increases in intensity. If the hypothesis that RGS19 regulates Wnt3a signaling is correct, overexpression of RGS19 would be expected to suppress this proximal step in the pathway, *i.e.*, Wnt3a-stimulated phosphorylation of Dvl3. Overexpression of RGS19 did not influence the pattern of Dvl3 phosphorylation in those F9 cells not treated with Wnt3a (Fig. 2.6A). In the Wnt3a-treated cells, however, overexpression of RGS19 sharply reduced the amount of the slower migrating phosphorylated form of Dvl3.

If the hypothesis that RGS19 exerts its inhibitory effects on Wnt/β-catenin signaling at the level of Gαo is correct, then activation of the pathway at a level
downstream of the G-protein should be resistant to RGS19 action. Expression of Dvl3 results in stimulation of Lef-Tcf-sensitive gene transcription (Fig. 2.6C). Expression of RGS19 is unable to block Dvl3-mediated transcription, adding further evidence that RGS19 inhibits Wnt action upstream of Dvl phosphorylation.

*Overexpression of RGS19 protein attenuates Wnt3a-induced formation of primitive endoderm*

Wnt3a and retinoic acid (RA) both stimulate F9 cells to form primitive endoderm (PE), an essential step in early stages of mouse development, through distinct signaling pathways. F9 cells promoted to PE no longer express embryonic markers, but rather express increased PE-specific markers, including cytokeratin endo A, recognized by the monoclonal TROMA antibody (Strickland and Mahdavi, 1978). These F9 cells form PE in response to Wnt3a or RA, demonstrated by an increase in the expression of the cytokeratin endo A marker (Fig. 2.7A). Cells transfected with an expression vector harboring RGS19 display a reduction in the PE-specific marker after Wnt3a stimulation (Fig. 2.7A). In contrast, RGS19 does not block the ability of RA to induce cytokeratin endo A expression and PE formation. Quantification of the data from several experiments indicates that overexpression of RGS19 attenuates Wnt3a-induced primitive endoderm formation by 50% (Fig. 2.7B), similar in magnitude to the suppression of signaling from Gαo to the level of β-catenin and to the level of induction of Lef-Tcf-sensitive gene transcription.

Indirect immunofluorescence was utilized to further characterize the expression of the cytokeratin endo A marker upon Wnt3a treatment (Fig. 2.7C). Untreated F9 cells do
not stain with the TROMA antibody. However, cells treated with Wnt3a showed an increase in fluorescence, indicating cytokeratin endo A expression and PE formation. Overexpression of RGS17 had no effect on the ability of Wnt3a to induce PE formation. However, cells expressing RGS19 were unable to form PE and showed decreased TROMA staining.

Knockdown of RGS19 attenuates Wnt3a-induced Lef-Tcf reporter activation and β-catenin accumulation

In order to further define the role of RGS19 in Wnt/β-catenin signaling, specific siRNA constructs were utilized to knockdown RGS19 protein expression in F9 cells. Cells treated with a “control” siRNA sequence provided by the commercial supplier displayed a robust Lef-Tcf transcriptional response to Wnt3a stimulation (Fig. 2.8A). In contrast, cells treated with an siRNA directed against RGS19 showed a greater than 50% decrease in Wnt3a-stimulated gene transcription. Two distinct siRNA sequences directed against RGS19 were tested, each with similar responses to Wnt3a-mediated gene transcription (data not shown). Western blot analysis confirmed that the specific siRNA directed against RGS19 was effective, reducing RGS19 protein levels to less than 10% that of control cells (Fig. 2.8B).

In order to confirm that the attenuation of Lef-Tcf-mediated transcription by RGS19 knockdown was dependent upon the Wnt/β-catenin pathway, we examined β-catenin stabilization in response to Wnt3a in cells treated with or without the RGS19 siRNA (Fig. 2.8C). Cells transfected with the control siRNA displayed a marked accumulation of cytosolic β-catenin 2 hr after Wnt3a stimulation, and β-catenin levels
remained stable past 4h. Knockdown of RGS19 countered the Wnt3a-mediated accumulation of β-catenin by 50%, at both 2 hr and 4hr timepoints while having no effect on basal β-catenin levels.

Since both overexpression and knockdown of RGS19 attenuated Wnt/β-catenin signaling, we hypothesized that perhaps some optimal level of RGS19 was necessary for maximal signaling output. In order to address this question, a “rescue” experiment was performed by adding increasing amounts of RNAi-resistant RGS19 cDNA to cells lacking endogenous RGS19 (Fig 2.8D). As shown above, transfection of F9 cells with siRNA directed against RGS19 attenuated Wnt3a-stimulated gene transcription by 50%. Addition of low levels of exogenous RGS19 (0.2 µg) was able to rescue the siRNA-mediated effect and restore wild-type levels of Wnt3a-induced gene transcription. However, increasing amounts of exogenous RGS19 attenuated gene transcription in a dose-dependent manner, highlighting the critical importance of the level of RGS19 expression in the regulation of Wnt/β-catenin signaling.

*RGS19 mutants attenuate Wnt3a-induced Lef-Tcf reporter activation*

Several amino acid resides within RGS19 have been identified as having critical roles in protein function. Serine 151 can be phosphorylated by Erk1/2, resulting in increased GAP activity (Ogier-Denis et al., 2000), and alanine 216 is required for maximal GIPC binding affinity (De Vries et al., 1998). In order to further investigate the complex functions of RGS19 in Wnt/β-catenin signaling, several RGS19 mutant constructs were generated and tested for the ability to impact Wnt3a-induced Lef-Tcf-mediated gene transcription (Fig. 2.9). The first construct, RGS19Δ216, involved
removal of the final amino acid of RGS19, and has been shown previously to ablate GIPC interaction (De Vries et al., 1998). Expression of RGS19Δ216 attenuated Wnt3a-stimulated gene transcription to a greater extent than wild-type RGS19. The next RGS19 mutant generated was RGS19S151A, in which the critical Erk1/2 phosphorylation site was changed to alanine. This construct has been shown previously to have impaired GAP activity (Ogier-Denis et al., 2000). Expression of RGS19S151A attenuated Wnt3a-induced Lef-Tcf-mediated gene transcription to a greater extent than wild-type RGS19.
Discussion

The Wnt/β-catenin pathway plays a critical role in development (Moon et al., 2004; Nusse, 2005) and dysregulation of this pathway can provoke diseases such as cancer (Reya and Clevers, 2005). Understanding the mechanisms by which cells respond to and modulate the Wnt downstream signals is therefore a focal point of investigation. Recent research has demonstrated that heterotrimeric G-proteins (i.e. Gαo) are essential transducers of the Wnt/β-catenin/Lef-Tcf pathway, from flies to mice (Katanaev et al., 2005; Liu et al., 2001; Liu et al., 2005; Malbon, 2004; Malbon, 2005; Slusarski et al., 1997a). That RGS proteins, which can regulate heterotrimeric G-proteins, may also regulate Wnt signaling was investigated herein. RGS proteins act as GTPase activating proteins (GAPs) for heterotrimeric G-proteins, increasing the intrinsic rate of GTP hydrolysis and accelerating signal termination (Hollinger and Hepler, 2002). RGS family members have essential roles in many physiological processes, including cardiovascular function (Heximer et al., 2003) and visual signal transduction (Chen et al., 2000). Earlier studies in Xenopus provided the first suggestion that RGS proteins may impact development (Wu et al., 2000). In this earlier study, injection of RNA encoding mouse RGS2 and RGS4 generated developmental defects similar to those observed following injection of a dominant negative mutant of XWnt-8 (Wu et al., 2000). Overexpression of RGS proteins RGS2 and RGS4 were also observed to block axis duplication resulting from injection of wild-type XWnt-8 (Wu et al., 2000). However, knockdown studies were not performed, precluding conclusive identification of the specific RGS proteins regulating Wnt signaling in this system. These observations can now be more fully
understood following demonstration of an obligate role of G-proteins in Wnt signaling (Malbon, 2005).

The current study tests the hypothesis that RGS19 protein regulates Wnt/β-catenin/Lef-Tcf signaling through inactivation of Gαo. RT-PCR was employed to ascertain the expression of mRNA for RGS proteins, focusing on the RZ subfamily member RGS19, in F9 mouse teratocarcinoma cells. RGS19, expressed in embryonic F9 cells, specifically attenuates Wnt3a-stimulated signaling, including Dvl phosphorylation, β-catenin accumulation, gene transcription and primitive endoderm formation. As G-proteins are known to be positive regulatory elements, our hypothesis was that RGS proteins, if involved, would counter the effects of Wnt3a on the Wnt/β-catenin/Lef-Tcf/PE pathway. We found that RGS19 overexpression, as well as its absence, suppressed the Wnt/β-catenin pathway.

Heterotrimeric G-proteins are molecular switches that cycle between active (GTP-bound) and inactive (GDP-bound) states through the action of specific guanine nucleotide exchange factors (Cabrera-Vera et al., 2003). Both positive and negative regulatory elements are required for control over G-protein signaling output. Upon ligand binding, G-protein coupled receptors (GPCRs) act as positive regulatory elements by exchanging the inhibitory GDP for GTP on the G-protein α-subunit. RGS proteins inhibit G-protein activation by stabilizing the transition state of the Gα-subunit for GTP hydrolysis, resulting in GDP production (Hollinger and Hepler, 2002). If RGS19 acts as a GAP for Gαo in the Wnt/β-catenin pathway, a rescue experiment can be performed through the use of a constitutively activated, mutant form of a G-protein (which cannot be regulated by its cognate RGS proteins). Indeed, cells transfected with Q205L Gαo, but not Q209L
Ga11or Q209L Gαq, were able to restore wild-type levels of Wnt-responsiveness after RGS19 inhibition.

Phosphorylation of Dvl is required for inhibition of the β-catenin degradation complex upon activation of the Wnt/β-catenin pathway (Yanagawa et al., 1995). Although the kinase responsible for Wnt-stimulated phosphorylation remains elusive, epistasis experiments place Dvl downstream of the heterotrimeric G-protein Gαo (Katanaev et al., 2005), and consequently, Dvl3 phosphorylation in response to Wnt3a. Expression of RGS19 was found to suppress Wnt3a-induced Dvl phosphorylation. In addition, RGS19 was not able to attenuate Dvl3-induced Lef-Tcf reporter activation, suggesting that RGS19 inhibition of Wnt/β-catenin signaling occurs upstream of Dvl.

Intracellular accumulation of β-catenin in response to Wnt3a stimulation is a defining feature of canonical Wnt signaling (Peifer et al., 1994). Cytosolic β-catenin enters the nucleus, binds to members of the Lef-Tcf family of transcription factors, and activates transcription of target genes (Molenaar et al., 1996). To confirm the specificity of the RGS19 inhibition of Lef-Tcf-mediated transcription, β-catenin accumulation in response to Wnt3a was measured in cells expressing RGS19. RGS19 expression diminished β-catenin accumulation in response to Wnt3a. This result agrees with the overarching hypothesis tested herein, i.e., that RGS19 regulates Wnt/β-catenin signaling upstream of β-catenin accumulation. Likewise, expression of RGS19 suppressed Wnt3a-induced PE formation in F9 cells, as measured by expression of the marker cytokeratin endo A.

Wnt/β-catenin signaling is mediated through the Lef-Tcf family of transcription factors (Molenaar et al., 1996). Lef-Tcf-sensitive signaling was measured through the
use of a luciferase-based reporter construct (Veeman et al., 2003). RGS19, but not other RGS proteins, attenuated gene reporter activation in a dose- and time-dependent manner. In order to probe the specificity of the RGS19-induced inhibition of Wnt3a signaling, a closely related RGS protein, RGS17, was also tested for its ability to alter Wnt3a-mediated gene transcription. Expression of RGS17 had no effect on Wnt3a-induced reporter activity. Therefore, although by *in vitro* reconstitution studies RGS17 has been shown to accelerate GTPase activity of several Gα proteins including Gαo, RGS19 is able (and RGS17 unable) to regulate Gαo in the context of Wnt3a/Frizzled1 signaling *in vivo*. This is not a surprising result, given our knowledge about RGS specificity. Early reports on RGS protein action by *in vitro* reconstitution revealed promiscuity of RGS proteins for G-protein α-subunits. RGS4 and RGS19, for example, were shown to act as GAPs *in vitro* for virtually all members of the Gαi family (Berman et al., 1996). The results from *in vivo* approaches appears to be more complex (Xie et al., 2005). RGS4 and RGS19 can regulate signaling through Gα-coupled opioid receptors (Xie et al., 2005). However, each RGS protein has been observed to display a distinct preference for regulating signaling through specific receptors, even when coupled to the same G-protein (Xie et al., 2005). Therefore, RGS protein action is not only a function of the G-protein α-subunit, but also of the GPCR whose action is being regulated and the cellular context that dictates proper stoichiometry of signaling elements.

RGS proteins negatively regulate G-protein signaling by acting as GAPs for heterotrimeric G-proteins. Therefore, knockdown of RGS proteins would be expected to result in increased signaling output. However, recent studies in *C. elegans* (Ferkey et al., 2007) and humans (Nishiguchi et al., 2004) have shown that loss of specific RGS
proteins can disrupt distinct G-protein mediated physiological events. In *C. elegans*,
chemosensation is controlled by G-protein-linked signaling cascades which are regulated
by RGS-3 (homologous to mammalian RGS8) (Ferkey et al., 2007). Interestingly,
animals lacking RGS-3 were unable to respond to strong sensory stimulation, despite
their normal role as negative regulators of this function. Similarly, light sensing in
vertebrates is mediated by G-proteins and regulated by a specific RGS protein, RGS9
(Chen et al., 2000). Humans lacking functional RGS9 protein display slow photoreceptor
deactivation, resulting in difficulty adjusting to rapid changes in light intensity
(Nishiguchi et al., 2004). Therefore, despite the role of RGS9 as an inhibitor of G-
protein signaling, loss of RGS9 also results in impaired visual signal transduction. In the
present study, we show that both the extremes of overexpression and of knockdown of
RGS19 protein attenuate Wnt3a/β-catenin signaling. Titration of RGS19 protein levels
through either siRNA-mediated knockdown or overexpression of RNAi-resistant RGS19,
however, highlights the critical importance of proper levels of RGS19 for maximal
Wnt/β-catenin signal transduction. In addition, overexpression or knockdown of the
RGS19-binding partner GIPC inhibits Wnt signaling in *Xenopus*, suggesting a common
necessity for proper physiological stoichiometry. That RGS proteins function as more
than simple negative regulators of G-proteins reflects the need for more detailed probes
into the complex roles for RGS19 in Wnt signal transduction.
Figure 2.1 - RGS protein expression in F9 cells: analysis at the mRNA level. (A) RT-PCR was performed on cDNA generated from RNA isolated from wild-type F9 cells. Primers specific for each RGS were utilized in the PCR reaction. PCR products were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide. RGS3, 5, 16, 10, 12, 14, 20, 17, 19, 9, 11, Axin and Conductin are expressed at the mRNA level in F9 cells. The lane labeled “No cDNA” shows that the PCR reaction is not contaminated with exogenous DNA.
Fig 2.1

A)
(B) Members of each RGS subfamily are expressed in F9 cells (R4, R12, RZ, R7, RA). All RGS proteins examined for mRNA expression are listed. Bold and italics indicates expression in F9 cells.
Fig 2.1

B)

RGS proteins

R4
RGS1
RGS2
RGS3
RGS4

R12
RGS5
RGS13
RGS16
RGS18

RZ
RGS10
RGS12
RGS14

R7
RGS20
RGS17
RGS19

RA
RGS6
RGS7
RGS9
RGS11

Axin
Conductin
Figure 2.2 - RGS protein overexpression screen. (A) F9 cells transiently transfected with Fz1, M50 and the indicated RGS proteins were treated with or without Wnt3a for 8 hr, then collected and subjected to luciferase assay. The results show that expression of RGS19 alone can significantly attenuate Wnt3a-induced gene transcription by more than 50%. * denotes $P < 0.05$ for the difference between wild-type cells and those expressing RGS19.
Fig 2.2

A)

![Bar graph showing transcriptional response (RLU) for different samples.

- The x-axis represents RGS (0.4mg DNA/well) with samples labeled as Con, 3, 5, 9, 10, 11, 12, 14, 16, 17, 19, and 20.
- The y-axis represents transcriptional response (RLU).
- Bars indicate the transcriptional response for each sample, with error bars showing variability.
- Sample 19 is marked with an asterisk, indicating a significant difference.

Transcriptional Response (RLU)

RGS (0.4mg DNA/well)
(B) Lysates were collected from F9 cells individually transiently transfected with an HA-tagged RGS protein. Each sample was subject to SDS-PAGE on an 11% acrylamide gel, transferred to nitrocellulose and probed with an antibody against HA. (C) Western blot analysis was used to determine the presence of RGS19 at the protein level in F9 cells. Wild-type lysates were separated by centrifugation into crude membrane and cytosolic fractions. Each sample was subject to SDS-PAGE on a 12.5% acrylamide gel, transferred to nitrocellulose and probed with an antibody against RGS19. In agreement with previous reports, one band is seen in the cytosolic fraction and two in the membrane fraction. Each fraction was treated with alkaline phosphatase, then subjected to SDS-PAGE on a 12.5% acrylamide gel, transferred to nitrocellulose and probed with an antibody against RGS19.
Fig 2.2

B)  

<table>
<thead>
<tr>
<th>HA-RGS</th>
<th>-</th>
<th>3</th>
<th>5</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>19</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_r$, kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C)  

<table>
<thead>
<tr>
<th>Untreated</th>
<th>AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyto</td>
<td>Mem</td>
</tr>
<tr>
<td>$M_r$, kDa</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

- p-RGS19
- RGS19
Figure 2.3 - RGS19 overexpression attenuates Wnt3a-induced Lef-Tcf reporter activation. (A) F9 cells transiently transfected with Fz1, M50 and RGS19 were treated with Wnt3a for the indicated lengths of time, then collected and subjected to luciferase assay. Overexpression of RGS19 blocked Wnt3a-induced reporter activation in a time-dependent manner. * denotes $P < 0.05$ for the difference between wild-type, Wnt3a-treated cells and those expressing RGS19 and Wnt3a-treated for the individual time points.
Fig 2.3

A) Transcription (RLU) over time with Wnt3a addition. The graph shows two curves: one for -RGS19 and one for +RGS19. The transcription levels peak at different times and are indicated with error bars.
(B) F9 cells transiently transfected with Fz1, M50 and the indicated amounts of RGS19 were treated with or without Wnt3a for 8 hr, then collected and subjected to luciferase assay. Inset, Western blot analysis was used to measure the expression of transiently transfected HA-tagged RGS19. Lysates were collected from cells transfected with the indicated amounts of HA-RGS19, then subjected to SDS-PAGE on a 12.5% acrylamide gel, transferred to nitrocellulose and probed with an antibody against HA. The results displayed are data from single experiments, representative of more than three independent tests. * denotes $P < 0.05$ for the difference between wild-type, Wnt3a-treated cells and those expressing RGS19 and Wnt3a-treated for the individual time points. ** denotes $P < 0.05$ for the difference between wild-type, untreated cells and those expressing RGS19 for the individual time points.
**Fig 2.3**

**B)**

Transcription (RLU) vs. RGS19 (µg DNA/well) with and without Wnt3a.

- **- Wnt3a**
- **+ Wnt3a**

Bar chart showing transcription values with significance levels indicated by asterisks. IB: Immunoblotting with HA-RGS19 and Actin markers.
(C) F9 cells transiently transfected with Fz1, M50 and the indicated amounts of RGS17 or RGS19 were treated with or without Wnt3a for 8 hr, then collected and subjected to luciferase assay. Results are displayed as difference in reporter activation upon Wnt3a stimulation as compared with untransfected control and are representative of three independent experiments. * denotes $P < 0.05$ for the difference between wild-type cells and those expressing RGS19 at each concentration. (D) Western blot analysis was used to measure the expression of transiently transfected HA-tagged RGS19 and RGS17. Lysates were collected from cells transfected with the indicated amounts of HA-RGS19 and HA-RGS17.
Fig 2.3

C)

Transcriptional Response (RLU)

\[ \text{cDNA (µg DNA/well)} \]

D)

RGS (mg DNA/well)  \[ 0 \quad 0.05 \quad 0.2 \quad 0.4 \]

\[ M_r, \text{ kDa} \]

HA-RGS17  \[ 28 \]

HA-RGS19  \[ 28 \]
Figure 2.4 - Constitutively active Gαo rescues inhibition of Wnt3a action by RGS19. (A) F9 cells transiently transfected with Fz1, M50 and/or RGS19, Q205L Gαo, Q209L Gα11 and Q209L Gαq were treated with or without Wnt3a for 8 hr, then collected and subjected to luciferase assay. The results show that expression of Q205L Gαo (left panel), but not Q209L Gα11 (right panel) or Q209L Gαq (bottom panel), can rescue the inhibitory effect of RGS19 on Wnt3a-mediated transcription. Results are displayed as difference in reporter activation upon Wnt3a stimulation as compared with untransfected control and are representative of three independent experiments.
Transcriptional Response (RLU)

RGS19: - - + +
GαoQL: - + - +

Gα11QL: - + - +

Fig 2.4
(B) Western blot analysis was used to measure the expression of transiently transfected Q205L Gαo, Q209L Gα11 and Q209L Gaq. Lysates were collected from cells transfected with Q205L Gαo, Q209L Gα11 Q209L Gaq, then subjected to SDS-PAGE on an 11% acrylamide gel, transferred to nitrocellulose and probed with an antibody against each G-protein. * denotes $P < 0.05$ for the difference between wild-type cells and those expressing either RGS19 or RGS19 and Q209L Gα11 or Q209L Gaq.
Fig 2.4

B)
Figure 2.5 - RGS19 overexpression attenuates β-catenin stabilization that results from Wnt3a stimulation. (A) F9 cells transiently transfected with Fz1 and either empty vector or RGS19 were treated with Wnt3a for the indicated lengths of time. Lysates were collected and treated with concanavalin A to separate cytosolic from membrane associated β-catenin. Cleared samples were subjected to SDS-PAGE on an 11% acrylamide gel, transferred to nitrocellulose and probed with an antibody against β-catenin. The increase in cytosolic β-catenin that is seen in response to Wnt3a is attenuated by overexpression of RGS19. The results displayed are from a single experiment representative of more than three independent tests. (B) Bands from multiple experiments were quantified by densitometry. * denotes $P < 0.05$ for the difference between wild-type cells and those expressing RGS19 for the individual time points.
Fig 2.5

A) 1B:

<table>
<thead>
<tr>
<th>Wnt3a (hr)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>- RGS19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ RGS19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$\beta$-catenin

$M_r$, kDa

B) + Wnt3a

![Bar graph showing intracellular $\beta$-catenin level](image)

Intracellular $\beta$-catenin level (density, arbitrary units)

Time (hr)
Figure 2.6 - RGS19 attenuates Wnt/β-catenin signaling upstream of Dvl. (A) F9 cells transiently transfected with Fz1 and RGS19 were treated with or without Wnt3a for the indicated amounts of time. Lysates were collected and separated by SDS-PAGE on a 10% acrylamide gel, transferred to nitrocellulose and probed with an antibody against Dvl3. The increase in phosphorylated Dvl3 that is seen in response to Wnt3a can be attenuated by overexpression of RGS19. (B) F9 cells transiently transfected with Fz1 and RGS19 were treated with or without Wnt3a for the indicated amounts of time. Lysates were collected, treated with alkaline phosphatase for 1 hr, separated by SDS-PAGE on a 10% acrylamide gel, transferred to nitrocellulose and probed with an antibody against Dvl3. The absence of the slowest migrating molecular weight band indicates that this band is a phosphorylated isoform of Dvl3.
Fig 2.6

A) Untreated samples

<table>
<thead>
<tr>
<th>Wnt3a (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>p-Dvl3</td>
</tr>
<tr>
<td>Dvl3</td>
</tr>
</tbody>
</table>

Control | RGS19
---|---

<table>
<thead>
<tr>
<th>Mr, kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>85</td>
</tr>
<tr>
<td>82</td>
</tr>
</tbody>
</table>

B) Alkaline phosphatase-treated samples

<table>
<thead>
<tr>
<th>Wnt3a (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>p-Dvl3</td>
</tr>
<tr>
<td>Dvl3</td>
</tr>
</tbody>
</table>

Control | RGS19
---|---

<table>
<thead>
<tr>
<th>Mr, kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>85</td>
</tr>
<tr>
<td>82</td>
</tr>
</tbody>
</table>
(C) F9 cells transiently transfected with Fz1 and Dvl3 or RGS19 were collected and subjected to luciferase assay. The increase in gene transcription stimulated by Dvl3 cannot be attenuated by RGS19. The results displayed are from a single experiment representative of more than three independent tests.
Figure 2.7 - Overexpression of RGS19 protein attenuates Wnt3a-induced formation of primitive endoderm. (A) F9 cells transiently transfected with Fz1, or Fz1 and RGS19 were treated with or without Wnt3a or retinoic acid (RA) for 5 days. Lysates were collected and separated by SDS-PAGE on an 11% acrylamide gel, transferred to nitrocellulose and probed with an antibody against cytokeratin endo A (TROMA), a specific marker for PE formation. Whereas cells expressing only Fz1 were able to differentiate upon Wnt3a stimulation, those containing both Fz1 and RGS19 were not. RGS19 was unable to block PE formation induced by RA treatment. The results displayed are from a single experiment representative of more than three independent tests. (B) Bands from multiple experiments were quantified by densitometry. Results are displayed as fold change in cytokeratin endo A expression as compared with untransfected control. * denotes \( P < 0.05 \) for the difference between untreated cells and those treated with either Wnt3a or RA.
Fig 2.7

A) 

Wnt3a

<table>
<thead>
<tr>
<th></th>
<th>- RGS19</th>
<th>+ RGS19</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Endo A

- 57

RA

- 57

B) 

- RGS19

+ RGS19

Endo A Expression

Control Wnt3a RA

*
(C) F9 cells transiently transfected with RGS17, RGS19 or an empty vector were treated with or without Wnt3a for 4 days. Cells were stained with an antibody against cytokeratin endo A and imaged by indirect immunofluorescence. Wnt3a treatment induced cytokeratin endo A expression in cells transfected with the empty vector or RGS17. Overexpression of RGS19 blocked cytokeratin endo A expression. Abbreviations: PC, phase contrast; OE, overexpression.
Fig 2.7

C)

<table>
<thead>
<tr>
<th></th>
<th>IF: TROMA</th>
<th>PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Untreated</td>
<td>+ Wnt3a</td>
</tr>
<tr>
<td>Control</td>
<td>+ Wnt3a</td>
<td>+ Wnt3a</td>
</tr>
<tr>
<td>RGS17 OE</td>
<td></td>
<td>+ Wnt3a</td>
</tr>
<tr>
<td>RGS19 OE</td>
<td></td>
<td>+ Wnt3a</td>
</tr>
</tbody>
</table>
Figure 2.8 - Knockdown of RGS19 protein attenuates Wnt3a-induced Lef-Tcf reporter activation. (A) F9 cells transiently transfected with an expression vector harboring siRNA sequences targeting RGS19 (or a control) and Fz1 and M50 were treated with or without Wnt3a for 8 hr, then collected and subjected to luciferase assay. * denotes $P < 0.05$ for the difference between cells transfected with the control siRNA vector and those transfected with the siRNA vector targeting RGS19. (B) Western blot analysis was used to measure the effect of siRNA treatment on RGS19 protein levels. Lysates were collected from cells transfected with each siRNA vector, then subjected to SDS-PAGE on a 12.5% acrylamide gel, transferred to nitrocellulose and probed with an antibody against RGS19. The siRNA targeting RGS19 was able to lower RGS19 protein expression to less than 10% that of control cells. The expression of actin was used as a loading control. The results displayed are data from single experiments, representative of more than three independent tests. (C) F9 cells transiently transfected with an expression vector harboring siRNA sequences targeting RGS19 (or a control) were treated with Wnt3a for the indicated lengths of time. Lysates were collected and treated with concanavalin A to separate cytosolic from membrane associated β-catenin. Cleared samples were subjected to SDS-PAGE on an 11% acrylamide gel, transferred to nitrocellulose and probed with an antibody against β-catenin. The increase in cytosolic β-catenin that is seen in response to Wnt3a is attenuated by knockdown of RGS19.
Fig 2.8

A) Transcriptional Response (RLU)

B) Western Blot

C) Western Blot
(D) F9 cells transiently transfected with Fz1, M50, the indicated amounts of RGS19 and an expression vector harboring siRNA sequences targeting RGS19 were treated with Wnt3a for 8 hr, then collected and subjected to luciferase assay. * denotes $P < 0.05$ for the difference between cells transfected with the control siRNA vector and those transfected with the siRNA vector targeting RGS19 alone or in combination with 0.5µg or 1µg of RGS19.
Fig 2.8

Transcriptional Response (RLU)

<table>
<thead>
<tr>
<th>RGS19 siRNA</th>
<th>-</th>
<th>+</th>
<th>+</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGS19 cDNA</td>
<td>-</td>
<td>-</td>
<td>0.2µg</td>
<td>0.5µg</td>
<td>1µg</td>
</tr>
</tbody>
</table>

* Significant difference
Figure 2.9 - RGS19 mutants attenuate Wnt3a-induced Lef-Tcf reporter activation. (A) F9 cells transiently transfected with Fz1, M50 and RGS19, RGS19Δ216, RGS19S151A or an empty vector were treated with or without Wnt3a for 8 hr, then collected and subjected to luciferase assay.
Transcriptional Response (RLU)
CHAPTER III

OSTM1 Regulates β-catenin/Lef1 Interaction and is Required for Wnt/β-catenin Signaling

Introduction

The canonical Wnt signaling cascade imposes tight regulation on the transcriptional co-activator β-catenin through a complex of proteins including GSK3β, Axin, and APC (Ikeda et al., 1998; Kishida et al., 1998; Zeng et al., 1997). Phosphorylation of β-catenin by protein kinases in this complex stimulates recognition and destruction of β-catenin by the proteasome (Aberle et al., 1997), ensuring low intracellular levels of “free” β-catenin in the absence of pathway activation. The Wnt/β-catenin pathway is triggered by binding of a Wnt ligand to Fz, a seven-transmembrane G-protein coupled receptor (Malbon, 2004), and subsequent downstream disruption of the β-catenin degradation complex through the action of heterotrimeric G-proteins and the phosphoprotein Dvl (Katanaev et al., 2005; Liu et al., 2001; Liu et al., 2005; Yanagawa et al., 1995). This allows β-catenin to accumulate in the cytosol, bind to members of the Lef/Tcf family of transcription factors, and activate transcription of genes necessary for both growth and differentiation (Molenaar et al., 1996). The Wnt/β-catenin pathway is required for a plethora of steps in the development of multicellular organisms and regulation of adult tissue homeostasis (Cleurs, 2006; Nusse, 2005), including the growth and renewal of bone (Krishnan et al., 2006). Several distinct steps in the accumulation of
bone mass are regulated by Wnt signaling, including stem cell self-renewal, inhibition of osteocyte apoptosis, and stimulation of osteoblast differentiation and proliferation (Krishnan et al., 2006). Defects in Wnt pathway components cause skeletal abnormalities, e.g., the osteoporosis-pseudoglioma disorder associated with mutations in Wnt co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) (Cheung et al., 2006).

Severe autosomal recessive osteopetrosis (Gong et al., 2001) is a genetic disease caused by deficiencies in bone resorption, resulting in increased bone mass. Patients with autosomal recessive osteopetrosis suffer from frequent fractures, hepatosplenomegaly and bone marrow obliteration, leading to early mortality unless treated with bone marrow transplantation (Quarello et al., 2004; Ramirez et al., 2004). Mutations in two human genes, TCIRG1 and CLCN7 (which encodes the ClC-7 protein), together are known to cause ~60% of autosomal recessive osteopetrosis cases. A spontaneous mouse model of ARO allowed the identification of a third candidate gene, osteopetrosis associated transmembrane protein 1 (OSTM1) (Chalhoub et al., 2003). Subsequently, several human mutations in OSTM1 have been uncovered in ARO patients (Quarello et al., 2004; Ramirez et al., 2004). OSTM1 is a type I transmembrane protein which localizes to intracellular vesicles (mainly endosomes and lysosomes) and homologs have been identified broadly, from humans to Caenorhabditis elegans and Drosophila melanogaster (Chalhoub et al., 2003). Two biological roles for OSTM1 have been suggested: serving as a cofactor necessary for ClC-7 function (Lange et al., 2006); and, functioning as an E3 ubiquitin ligase for the heterotrimeric G-protein Gαi3 (Fischer et al., 2003). As Wnt
signaling and OSTM1 are both intimately involved in bone remodeling, we explored the hypothesis that OSTM1 plays a role in Wnt/β-catenin signal transduction.
**Materials and Methods**

**Plasmids and antibodies**

An expression vector encoding human OSTM1 was obtained from OriGene (Rockville, MD). Expression vectors containing Fz1 and M50 were provided by Dr. Randall Moon (Department of Pharmacology, HHMI, University of Washington, Seattle, WA). Expression vectors containing SAβ-cat and β-cat/Lef were provided by Dr. Ken-Ichi Takemaru (Department of Pharmacology, SUNY Stony Brook, Stony Brook, NY) and an expression vector harboring VP16/Lef was provided by Dr. Howard Crawford (Department of Pharmacology, SUNY Stony Brook, Stony Brook, NY). The monoclonal TROMA-1 antibody was generated by the University of Iowa Developmental Studies Hybridoma Bank (Iowa City, IA). The rabbit antibody against β-catenin and the mouse antibody against actin were obtained from Sigma (Saint Louis, MO) and the anti-HA affinity matrix was obtained from Roche (Indianapolis, IN).

**Cell culture and transfection**

Mouse F9 teratocarcinoma cells were obtained from the ATCC (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, penicillin (60 μg/ml) and streptomycin (100 μg/ml) in a humidified atmosphere chamber supplied with 5% CO₂ at 37° C. Cells were transfected by using Lipofectamine (Invitrogen, Carlsbad, CA), according to the manufacturer’s suggested protocol. Pre-designed siRNA oligonucleotides targeting OSTM1 were obtained from Ambion (Austin, TX). The sequences are as follows: Sense: ccaaaaaauuacccgaagtt; Antisense: cuucggagauuuuuuggg.
The annealed siRNA sequences were transfected into F9 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer’s suggested protocol. RNA was collected 72h after transfection and knockdown efficiency measured by RT-PCR.

**Reverse-transcription polymerase chain reaction**

Specific primers for amplification of regions of OSTM1 and Cyclophilin A were obtained from Operon (Huntsville, AL). The sequences are as follows: OSTM1: atggctcgggacgcggag, aggcaatttgcgcagttc gcc; CycA: agcactggagagaaaggatttg, cacaatgttcatgcctttc. RNA was isolated from wild-type F9 cells using the RNA STAT-60 reagent (Tel-Test, Friendswood, TX) according to the manufacturer’s suggested protocol. The reverse transcription reaction was performed using the Invitrogen Superscript system according to the manufacturer’s suggested protocol. Primer sets (0.5 µM) were mixed with dNTPs (1 mM), F9 cDNA (100 ng), 10X *Pfu* buffer and *Pfu Turbo* DNA polymerase (Stratagene, La Jolla, CA). The PCR reaction was performed using the GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA) with the following protocol: 95°C, 5 minutes; (95°C, 1 minute; 60°C, 1 minute; 72°C, 3 minutes) 30 cycles; 72°C, 15 minutes. Reaction products were resolved electrophoretically upon 1% agarose gels and stained with ethidium bromide.

**Gene transcription assay**

F9 cells were seeded into 12-well plates and transiently transfected as described above. After incubation for 48 hours at 37°C, cells were serum-starved overnight, then treated with purified Wnt3a (R&D Systems, Minneapolis, MN). Cells were lysed
through addition of diluted Cell Culture Lysis Reagent 5X (Promega, Madison, WI). Lysates were collected and centrifuged at 15,000 x g for 5 minutes. The supernatant was directly assayed as described below. A sample (20 µl) of lysate was added to 100 µl of luciferase assay buffer (20 mM Tricine, pH 7.8, 1.07 mM MgCO₃, 4 mM MgSO₄, 0.1 mM EDTA, 0.27 mM coenzyme A, 0.67 mM luciferin, 33.3 mM DTT, 0.66 µM ATP) and a luminometer (Berthold Lumat LB 9507) was used to measure luciferase activity.

_Cytosolic β-catenin assay_

In order to separate cytosolic from membrane-associated β-catenin, samples were treated with concanavalin A (Con A). Con A (covalently linked to Sepharose) was obtained from Amersham Biosciences (Upsala, Sweden). F9 cells were washed once with PBS and lysed in RIPA buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) plus leupeptin (10 µg/mL), aprotinin (10 µg/mL) and PMSF (200 µM). Lysates were rotated at 4° C for 20 minutes and then centrifuged for 25 minutes at 15,000 rpm. The protein concentration of the supernatant fraction was determined and lysates were diluted with RIPA buffer to a protein content of 2.5 mg/ml. Sixty µl of Con A-Sepharose was added to each sample which then was incubated at 4° C for 1 hour with rotation. The supernatant was then removed and the protein concentration determined. The samples were subjected to SDS-PAGE on 10% acrylamide gels, the separated proteins transferred to nitrocellulose blots, and the blots probed with the anti-β-catenin antibody.
**Indirect immunofluorescence**

F9 cells grown in 24-well plates were fixed in 3% paraformaldehyde, then washed three times in MSM-PIPES buffer (18 mM MgSO₄, 5 mM CaCl₂, 40 mM KCl, 24 mM NaCl, 5 mM PIPES, 0.5% Triton X-100, 0.5% NP40). Cells were incubated at 37°C for 30 minutes with the TROMA antibody, washed with MSM-PIPES, and then incubated at 37°C for 30 minutes with an anti-mouse antibody coupled to Alexa Flour 488 (Invitrogen). Cells were then washed in blotting buffer (560 mM NaCl, 10 mM KPO₄, 0.1% Triton X-100, 0.02% SDS) and imaged with a Zeiss LSM510 inverted fluorescence microscope.

**Immunoprecipitation**

F9 cells grown in 100 mm plates were lysed in a buffer composed of 50 mM TrisHCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.5% NP-40. 500 µg protein was incubated with 50 µl of resuspended Anti-HA Affinity Matrix overnight at 4°C. The matrix was washed three times in lysis buffer and the bound proteins were eluted with sample buffer. The samples were subjected to SDS-PAGE on 10% acrylamide gels, the separated proteins transferred to nitrocellulose blots, and the blots probed with the anti-β-catenin antibody.
Results

OSTM1 mRNA is expressed in F9 cells

We chose to investigate the putative role of OSTM1 in Wnt/β-catenin signaling in mouse teratocarcinoma cells (F9). These pluripotent cells have been used as a model system to study signaling events in early mouse development and can be differentiated to primitive endoderm (PE) by stimulation with Wnt3a (Liu et al., 2001). The lack of suitable antibodies precluded determination by immunoblotting of OSTM1 protein expression in the mouse F9 cells. Alternatively, we sought confirmation at the level of mRNA, using reverse-transcription polymerase chain reaction (RT-PCR). OSTM1 mRNA was positively detected, being expressed in these cells (Fig. 3.1).

OSTM1 potentiates Wnt/β-catenin signaling

The transcriptional response to Wnt/β-catenin signaling can be measured through the use of an Lef/Tcf-responsive promoter driving luciferase expression (M50) (Veeman et al., 2003). Treatment of wild-type F9 cells with purified Wnt3a stimulated a ~10-fold increase in gene transcription, as measured by luciferase activity (Fig. 3.2A). We overexpressed OSTM1 in F9 cells in order to probe whether or not OSTM1 has a functional impact on Wnt/β-catenin signaling. Overexpression of OSTM1 increased the Wnt3a-induced Lef/Tcf response in a dose-dependent manner (Fig. 3.2A). The potentiation of Wnt3a-stimulated activity was more than double in those cells transfected with the highest amount of expression vector harboring OSTM1. The basal levels of reporter activation also were potentiated by overexpression of OSTM1. Cytosolic accumulation of β-catenin and subsequent nuclear translocation are required for Wnt-
responsive Lef/Tcf-mediated gene transcription (Noordermeer et al., 1994). Therefore, we probed the intracellular β-catenin levels in Wnt3a-treated cells, comparing those cells that were overexpressing OSTM1 to those transfected with an empty vector (Fig. 3.2B). Intracellular levels of β-catenin were low in unstimulated F9 cells (Fig. 3.2B; 0 hr time points). Upon Wnt3a stimulation of cells transfected with an empty vector, accumulation of β-catenin was seen within 2 hours, reaching a peak by 4 hours and declining thereafter. In cells expressing OSTM1, basal levels of intracellular β-catenin were unchanged as compared with the control condition. However, in cells transfected with OSTM1 and treated with Wnt3a, accumulation of β-catenin occurred rapidly (within 1 hour), and was maintained at an elevated level past 5 hours.

OSTM1 knockdown attenuates Wnt/β-catenin signaling

If OSTM1 acts to potentiate Wnt3a stimulation of the canonical pathway, conventional wisdom would suggest that the absence of OSTM1 would also impact signaling. To probe this role of OSTM1 in Wnt/β-catenin signaling, specific siRNA sequences were utilized to knock down OSTM1 expression in F9 cells. Cells transfected with a control (scrambled) siRNA sequence responded to Wnt3a stimulation with a normal ~10-fold increase in Lef/Tcf-mediated gene transcription (Figs. 3.2A, 3.3A). In the absence of Wnt3a, basal levels of gene transcription were equivalent in control and OSTM1 knockdown conditions (Fig. 3.3A). The Lef/Tcf-mediated transcriptional response to Wnt3a, in sharp contrast, was attenuated by more than 70% in cells treated with an siRNA targeting OSTM1 (Fig. 3.3A). OSTM1 appears to play an essential role in Wnt/β-catenin signaling in response to Wnt3a. RT-PCR analysis of the levels of
OSTM1 mRNA demonstrated that the siRNA reagents effectively knocked down OSTM1 mRNA (Fig. 3.3B). Two distinct siRNA sequences designed by the commercial supplier to target OSTM1 were utilized in these experiments. Treatment with either of these siRNAs sharply attenuated the ability of Wnt3a to activate Lef/Tcf-sensitive transcription (data not shown).

A rescue experiment was performed to test the specificity of the OSTM1 knockdown response. As described above, knockdown of OSTM1 with siRNA sharply attenuated Wnt3a-mediated transcription (Fig. 3.3C). Overexpression of human OSTM1 cDNA in these siRNA knockdown cells lacking endogenous OSTM1 successfully rescued the ability of these cells to respond to Wnt3a. The rescue of the Wnt3a-induced canonical pathway in OSTM1-depleted cells by overexpression of OSTM1 was dose-dependent with regard to the expression vector harboring OSTM1 (Fig. 3.3C).

\[
\text{OSTM1}^\Delta C \text{ mutant acts as a dominant negative in Wnt/\beta-catenin signaling}
\]

Having demonstrated a functional role of OSTM1 in Wnt/\beta-catenin canonical signaling we were able to focus on the effects of a known OSTM1 mutation that causes osteopetrosis in humans. There are multiple mutations of \textit{OSTM1} that cause autosomal recessive osteopetrosis (Chalhoub et al., 2003). We investigated the effect of a well-known human \textit{OSTM1} mutation (Lange et al., 2006) on Wnt/\beta-catenin signaling. The OSTM1 C-terminal deletion mutant (deletion of the final 72 amino acids; OSTM1\Delta C) was constructed and we tested its ability to modulate Wnt/\beta-catenin canonical signaling. Overexpression of OSTM1\Delta C was found to attenuate Wnt3a-induced transcription in a dose-dependent manner (Fig. 3.4A). Basal levels of Lef/Tcf-sensitive transcription were
influenced to a lesser extent. Next, a time-course for Wnt3a-mediated gene transcription was undertaken (Fig. 3.4B). Treatment of F9 cells with Wnt3a induced an increase in Lef/Tcf-sensitive transcription by four hours, rising sharply at five through eight hours. Expression of OSTM1∆C attenuated the amplitude of Wnt3a-induced transcriptional response by more than 50% (Fig. 3.4B).

To focus on the point at which the OSTM1∆C mutant impacts the Wnt-induced transcriptional pathway, we measured β-catenin levels in cells expressing OSTM1∆C or an empty vector and treated with Wnt3a (Fig. 3.4C). Wild-type F9 cells treated with Wnt3a initiated accumulation of cytosolic β-catenin within 2 hr, reached a peak of β-catenin around 3-4 hr, and declining thereafter. Cells expressing OSTM1∆C displayed no change in basal levels of cytosolic β-catenin. Expression of OSTM1∆C clearly attenuated the ability of Wnt3a to induce β-catenin accumulation (Fig. 3.4C).

The effects of expression of OSTM1∆C on Lef/Tcf-mediated transcription were measured in response to activation of the canonical pathway by Wnt3a and downstream elements in order to determine the epistasis of OSTM1∆C action on Wnt signaling (Fig. 3.4D). Expression of OSTM1∆C inhibited Wnt3a-induced transcription by more than 50% (Fig. 3.4A,B,D). Overexpression of Dvl3 induces Lef/Tcf-mediated gene transcription (Feigin and Malbon, 2007). OSTM1∆C expression inhibited Dvl3-stimulated transcriptional activation by 50% (Fig. 3.4D). Expression of constitutively active β-catenin mutant (lacking four N-terminal phosphorylation sites necessary for proteasome recognition; SAβ-cat) was utilized to activate Lef/Tcf gene transcription. Overexpression of OSTM1∆C attenuated SAβ-cat-induced transcriptional activation by more than 70% (Fig. 3.4D). Expression of a β-catenin/Lef fusion protein (β/Lef) (Filali
et al., 2002) as well as a constitutively-active Lef mutant (VP16/Lef) (Aoki et al., 1999) were examined to explore further downstream signaling events in the Wnt/β-catenin pathway. In contrast to its ability to attenuate canonical signaling in response to activation by Wnt3a, overexpression of Dvl3, and expression of SAβ-catenin, expression of OSTM1ΔC was unable to attenuate Lef/Tcf-mediated signaling induced by expression of either of the Lef mutants (Fig. 3.4D).

To further explore the ability of OSTM1 to inhibit Wnt signaling, we tested the effects of OSTM1 expression on cells with increasing expression of SAβ-catenin (Fig. 3.4E). In the absence of expression of SAβ-catenin OSTM1ΔC expression had no obvious effect on Lef/Tcf-sensitive transcription, when compared with cells transfected with an empty vector. At higher levels of expression of SAβ-catenin, however, OSTM1ΔC-induced inhibition was observed. Expression of OSTM1ΔC inhibited transcription ~50%, even at the highest tested levels of SAβ-catenin.

OSTM1ΔC attenuates the ability of Wnt3a to stimulate PE formation

F9 cells can be induced to form PE in response to Wnt3a (Liu et al., 2001), indicated by the expression of PE-specific markers such as cytokeratin endo A. PE is a cardinal stage in early mouse development and can be measured using the mouse TROMA antibody to endo A (Strickland and Mahdavi, 1978). Indirect immunofluorescence was utilized to detect expression of cytokeratin endo A in F9 cells treated with Wnt3a (Fig. 3.5). Untreated F9 cells do not express cytokeratin endo A, i.e., no positive staining by the TROMA antibody. Expression of OSTM1ΔC blocked the ability of F9 cells to form PE in response to Wnt3a. In the absence of Wnt3a,
overexpression of OSTM1 or OSTM1\(\Delta C\) had no effect on TROMA staining. In the presence of Wnt3a, cells transfected with an empty vector or OSTM1 stained positive for TROMA, indicating PE formation. Thus, expression of the OSTM1\(\Delta C\) mutant blocked the ability of Wnt3a to signal to a major embryonic response, PE formation.

**OSTM1 regulates \(\beta\)-catenin/Lef1 interaction**

The epistasis experiments (Fig. 3.4C) implicated OSTM1\(\Delta C\) action on Wnt/\(\beta\)-catenin signaling, downstream of \(\beta\)-catenin accumulation, but upstream of \(\beta\)-catenin/Lef1 interaction. We compared the ability of OSTM1 and OSTM1\(\Delta C\) to regulate the Wnt3a-induced binding of \(\beta\)-catenin to Lef1 (Fig. 3.6). In the absence of Wnt3a, immunoprecipitated HA-Lef1 was able to pull down a small amount of endogenous \(\beta\)-catenin. This effect was unaltered in unstimulated cells expressing OSTM1 or OSTM1\(\Delta C\). \(\beta\)-catenin binding to Lef1 increased in cells treated with Wnt3a (Fig. 3.6, compare lanes 1 and 2). The ability of \(\beta\)-catenin to bind Lef1 was increased in Wnt3a-treated cells expressing OSTM1. In Wnt3a-treated cells expressing OSTM1\(\Delta C\), in sharp contrast, no detectable binding of \(\beta\)-catenin was observed.
Discussion

The Wnt/β-catenin pathway controls numerous steps both in the development and maintenance of multicellular organisms (Clevers, 2006; Nusse, 2005). Importantly, Wnt signals modulate bone formation through regulation of stem cell self-renewal, osteocyte apoptosis and osteoblast differentiation and proliferation (Krishnan et al., 2006). Mutations in several Wnt signaling components lead to dramatic bone defects, including osteoporosis-pseudoglioma in patients with LRP5 defects (Cheung et al., 2006).

ARO is a severe genetic disorder caused by mutations in several genes affecting osteoclast function, leading to fragile bones and increased infection rates (Quarello et al., 2004; Ramirez et al., 2004). Recently, defects in the OSTM1 gene have been found to generate ARO, both in humans and in the spontaneous grey-lethal mouse model. While several functions for OSTM1 have been proposed (Fischer et al., 2003; Lange et al., 2006), the exact mechanism whereby defects in this protein lead to ARO warrant further investigation. As Wnt signaling is critically important for bone maintenance, in the present study we tested the hypothesis that OSTM1 regulates Wnt/β-catenin signaling. We found that OSTM1 overexpression potentiated, and OSTM1 knockdown suppressed, Wnt3a-mediated responses in F9 cells. Furthermore, we determined the mechanism whereby OSTM1 regulates Wnt signaling: through modulation of the β-catenin/Lef1 interaction, a crucial downstream event in Wnt/β-catenin signal transduction.

In order to assess the effect of OSTM1 overexpression or suppression on Wnt/β-catenin signaling we utilized a luciferase-based Lef/Tcf-sensitive reporter construct which can be activated in response to Wnt3a or transfection with constitutively active Wnt signaling components (Veeman et al., 2003). Overexpression of OSTM1 intensified
Intracellular accumulation of β-catenin in response to Wnt3a is a hallmark of the canonical Wnt signaling pathway (Peifer et al., 1994) and can be used as a reliable marker of Wnt3a signaling activity. Overexpression of OSTM1 intensified β-catenin accumulation in response to Wnt3a, while overexpression of OSTM1∆C attenuated this response. Similarly, expression of OSTM1∆C inhibited Wnt3a-mediated PE formation. These results are consistent with the transcriptional readouts and suggestive of a positive role for OSTM1 in Wnt/β-catenin signaling.

In order to determine the mechanism of action of OSTM1 in the Wnt signal transduction pathway, knowledge of the epistatic level of OSTM1 was first required. Epistasis experiments revealed that OSTM1∆C blocked Wnt/β-catenin signaling downstream of β-catenin accumulation, yet upstream of the transcriptionally active β-catenin/Lef1 protein complex. Therefore, we tested the ability of OSTM1 and OSTM1∆C to regulate the β-catenin/Lef1 interaction. Overexpression of OSTM1 intensified, while OSTM1∆C inhibited, the Wnt3a-stimulated interaction of β-catenin with Lef1, suggesting that OSTM1 may function as a mediator of this critical protein complex. It will be informative to probe the β-catenin/Lef1 interaction in osteoclasts from grey-lethal mice or ARO patients with OSTM1 deficiency. Furthermore, little is known about the role of Wnt signaling in osteoclast maturation and function. Our results
suggest that mutations in OSTM1 may contribute to ARO through disruption of Wnt/β-catenin signaling.
Figure 3.1 - OSTM1 mRNA is expressed in F9 cells. RT-PCR was performed on cDNA generated from RNA isolated from wild-type F9 cells. Primers specific for OSTM1 and Cyclophilin A were utilized in the PCR reaction. PCR products were separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide. The lane labeled “No cDNA” shows that the PCR reaction is not contaminated with exogenous DNA.
Fig 3.1
Figure 3.2 - OSTM1 potentiates Wnt/β-catenin signaling. (A) F9 cells transiently transfected with Fz1, M50 and the indicated amounts of OSTM1 were treated with Wnt3a for 7 hr, then collected and subjected to luciferase assay. * denotes $P < 0.05$ for the difference between wild-type, Wnt3a-treated cells and those expressing OSTM1 and Wnt3a-treated for the individual doses. The data shown are mean values (+/- s.e.) of at least three separate experiments. Lower panel: immunoblotting (IB) western blot analysis was used to measure the expression of transiently transfected HA-tagged OSTM1.
Fig 3.2

A) Lef/Tcf-sensitive transcription (RLU)

- Wnt3a
+ Wnt3a

OSTM1 (µg DNA/well)

OSTM1

WB: HA
(B) F9 cells transiently transfected with Fz1 and either empty vector or OSTM1 were treated with Wnt3a for the indicated lengths of time. Lysates were collected and treated with concanavalin A to separate cytosolic from membrane associated β-catenin. Cleared samples were subjected to SDS-PAGE on an 11% acrylamide gel, transferred to nitrocellulose and probed with an antibody against β-catenin. The results displayed are from a single experiment representative of more than three independent tests.
B)

<table>
<thead>
<tr>
<th>Expression vector:</th>
<th>Empty</th>
<th>OSTM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt3a (hr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 1 2 3 4 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-catenin</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Actin</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 3.3 - OSTM1 knockdown attenuates Wnt/β-catenin signaling. (A) F9 cells transiently transfected with siRNA sequences targeting OSTM1 (or a control sequence) and expression vectors encoding Fz1 and M50 were treated with or without Wnt3a for 7 hr, then collected and subjected to luciferase assay. * denotes $P < 0.05$ for the difference between cells transfected with the control siRNA and those transfected with the siRNA targeting OSTM1. (B) Reverse-transcription PCR analysis was used to measure the effect of siRNA treatment on OSTM1 mRNA levels. The expression of cyclophilin A was used as a loading control.
Fig 3.3

A) Lef/Tcf-sensitive transcription (RLU)

B) siRNA: Con OSTM1

- [Image of bar graph showing Lef/Tcf-sensitive transcription levels with - Wnt3a and + Wnt3a conditions]

- [Image of gel showing OSTM1 and CycA bands]
(C) F9 cells transiently transfected with siRNA sequences targeting OSTM1 and expression vectors encoding Fz1, M50 and the indicated amounts of OSTM1 were treated with or without Wnt3a for 7 hr, then collected and subjected to luciferase assay. * denotes $P < 0.05$ for the difference between cells transfected with the OSTM1 siRNA and those transfected with the siRNA targeting OSTM1 and OSTM1 cDNA.
Fig 3.3

Lef/Tcf-sensitive transcription (RLU)
Figure 3.4 - OSTM1ΔC mutant attenuates Wnt/β-catenin signaling. (A) F9 cells transiently transfected with Fz1, M50 and either empty vector or the indicated amounts of OSTM1ΔC were treated with Wnt3a for 7 hr, then collected and subjected to luciferase assay. * denotes $P < 0.05$ for the difference between wild-type, Wnt3a-treated cells and those expressing OSTM1ΔC and Wnt3a-treated for the individual doses. Lower panel: IB western blot analysis was used to measure the expression of transiently transfected HA-tagged OSTM1ΔC.
Fig 3.4

A)

Lef/Tcf-sensitive transcription (RLU)

OSTM1ΔC (µg DNA/well)

0 0.2 0.5 1

OSTM1ΔC

WB: HA
(B) F9 cells transiently transfected with Fz1, M50 and either empty vector or OSTM1ΔC were treated with Wnt3a for the indicated lengths of time, then collected and subjected to luciferase assay.
**B)** Lef/Tcf-sensitive transcription (RLU)

- 0 2 4 6 8
- OSTM1
+ OSTM1ΔC

Time (hr)

0 2 4 6 8

Lef/Tcf-sensitive transcription (RLU)
(C) F9 cells transiently transfected with Fz1 and either empty vector or OSTM1ΔC were treated with Wnt3a for the indicated lengths of time. Lysates were collected and treated with concanavalin A to separate cytosolic from membrane associated β-catenin. Cleared samples were subjected to SDS-PAGE on an 11% acrylamide gel, transferred to nitrocellulose and probed with an antibody against β-catenin. The results displayed are from a single experiment representative of more than three independent tests.
Fig 3.4

C)

<table>
<thead>
<tr>
<th>Expression vector:</th>
<th>Empty</th>
<th>OSTM1ΔC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt3a (hr)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>β-cat</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Actin</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-cat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(D) F9 cells transiently transfected with Fz1, M50 and the indicated constructs (or treated with Wnt3a for 7 hr) were collected and subjected to luciferase assay. * denotes $P < 0.05$ for the difference between Wnt/β-catenin pathway activated cells and those expressing OSTM1ΔC.
Fig 3.4

D) - OSTM1ΔC + OSTM1ΔC

Lef/Tcf-sensitive transcription (RLU)

Wnt3a

Dvl3

SAβ-cat

β-cat/Lef

VP-16/Lef
(E) F9 cells transiently transfected with Fz1, M50, OSTM1ΔC and the indicated amounts of SAβ-catenin were collected and subjected to luciferase assay. * denotes $P < 0.05$ for the difference between cells transfected with SAβ-catenin and an empty vector and those transfected with SAβ-catenin and OSTM1ΔC. Lower panel: IB western blot analysis was used to measure the expression of transiently transfected myc-tagged SAβ-catenin.
Fig 3.4

E)

SAβ-cat (µg/well)

SAβ-cat

WB: myc
Figure 3.5 - OSTM1ΔC attenuates Wnt3a-induced PE formation. F9 cells transiently transfected with OSTM1, OSTM1ΔC or an empty vector were treated with or without Wnt3a for 4 days. Cells were stained with an antibody against cytokeratin endo A and imaged by indirect immunofluorescence. PC, phase contrast.
Fig 3.5

<table>
<thead>
<tr>
<th></th>
<th>- Wnt3a</th>
<th></th>
<th>+ Wnt3a</th>
</tr>
</thead>
<tbody>
<tr>
<td>IF: TROMA</td>
<td>PC</td>
<td>IF: TROMA</td>
<td>PC</td>
</tr>
<tr>
<td>Control (EV)</td>
<td></td>
<td>OSTM1 WT</td>
<td></td>
</tr>
<tr>
<td>OSTM1ΔC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Image of IF: TROMA PC control (EV) OSTM1 WT OSTM1ΔC]
Figure 3.6 - OSTM1 regulates β-catenin/Lef1 interaction. F9 cells transiently transfected with Lef1-HA and either OSTM1, OSTM1ΔC or an empty vector were treated with or without Wnt3a for 3 hours. Cell lysates were collected and subjected to immunoprecipitation with an anti-HA antibody. The amount of endogenous β-catenin pulled down under each condition was measured through Western blot analysis using an anti-β-catenin antibody.
Fig 3.6

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>OSTM1</th>
<th>OSTM1ΔC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt3a</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>IP: HA-Lef1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB: β-cat</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

WB: β-catenin
The Wnt/β-catenin signal transduction cascade is a critical regulator of mammalian embryogenesis and adult tissue homeostasis. Mutations in Wnt pathway components lead to abnormal development and are associated with several disease states, including cancer, and defects in bone maintenance. Hundreds of studies over 20 years have identified the key protein components of the Wnt/β-catenin pathway. However, the exact mechanism by which binding of a Wnt ligand to a Frizzled receptor triggers eventual β-catenin-dependent gene transcription is largely unclear. For example, it is unknown how activation of the G-proteins, Gαo and Gαq, leads to phosphorylation of Dvl. Also uncertain is how hyperphosphorylated Dvl disrupts the β-catenin degradation complex consisting of Axin and APC. Further upstream, how does the interaction of a specific Wnt with a specific Frizzled selectively activate one or more of the three Wnt signaling pathways? What role does LRP5/6 play in this decision?

Dozens of regulatory molecules have been reported to modulate Wnt signaling, either directly, or through crosstalk with distinct signaling pathways. For example, several secreted proteins act as canonical pathway antagonists, including Dickkopf, which binds to LRP5/6 (Glinka et al., 1998) and sRFP, which directly binds to Wnt (Hoang et al., 1996). These molecules act in a specific spatial and temporal manner to
influence the diffusion of the Wnt signal away from the sending cell. Crosstalk between the Wnt/β-catennin pathway and several signaling cascades, including Ras-ERK (Jeon et al., 2007) and Notch (Nakamura et al., 2007), has been reported. How these distinct protein pathways form a tightly regulated network of signaling interactions allowing proper embryonic growth and development is a key question for further study.

Due to the almost universal role of Wnt/β-catenin signaling in mammalian development and homeostasis, as well as stem cell biology, it has been a challenge to directly target this pathway effectively in conditions of aberrant activation, such as cancer. However, the pharmacological inhibition of regulatory molecules, which may not be required in every cell type or under every cellular condition, can open the door for more specific disease therapies. This thesis sought to identify and characterize novel Wnt/β-catenin regulatory molecules, with the goal of developing new drug targets for the modulation of this critically important signal transduction cascade.

I began my search for novel Wnt signaling components by focusing on the heterotrimeric G-protein, Gαo. This GTPase is required for the Wnt/β-catenin pathway, downstream of the G-protein coupled receptor Frizzled and upstream of Dvl. I reasoned that tight control over pathway activation might be accomplished through the utilization of RGS proteins, which act as GAPs for heterotrimeric G-proteins and regulate many physiologically important processes, including cardiac function and vision. Interestingly, Axin, a critical negative regulator of Wnt signaling, contains an RGS domain. However, this region of the protein is used to bind APC and appears to have lost functional GAP activity. Therefore, I turned my attention to the other ~30 mammalian RGS-domain containing proteins. Through a systematic investigation I uncovered RGS19 as a novel
regulator of Wnt/β-catenin signaling. Overexpression analysis revealed that RGS19 specifically attenuated several Wnt-induced responses, including Dvl3 phosphorylation, β-catenin accumulation, Lef/Tcf-mediated gene transcription and PE formation. Furthermore, RGS19 was shown to act downstream of Gaα, yet upstream of Dvl3 in the canonical pathway. Finally, knockdown of RGS19 also attenuated Wnt signaling, suggesting a complex role for this protein in Wnt/β-catenin signal transduction.

This data generates several interesting questions and future avenues of research. For example, how is RGS19 specifically targeted to the Frizzled-1 receptor? Is GIPC required for this interaction? Mutation of the RGS19 PDZ-binding ligand, which ablates GIPC association, impairs Wnt/β-catenin signaling to a greater extent than wild-type RGS19. This data suggests that a Frizzled/RGS19/GIPC complex is required for optimal signaling activity. Future studies into the trafficking of all three molecules by confocal microscopy will be necessary to further understand the regulation of RGS19 activity.

What do these results tell us about the utility of RGS19 as a possible drug target for the Wnt/β-catenin pathway? Although RGS proteins do not act through a catalytic mechanism, they are still amenable to pharmacological inhibition by molecules that disrupt their binding to G-proteins (Jin et al., 2004a; Jin et al., 2004b; Roof et al., 2006). To date, several inhibitors of RGS4 have been developed and are being tested in tandem with GPCR agonists to further enhance G-protein mediated signaling. As RGS19 knockdown attenuates Wnt signaling, it would be informative to measure the ability of RGS19 inhibitors to modulate pathway activity. However, because RGS19 is widely expressed in mammalian tissues and inhibits G-protein signaling through many GPCRs, its utility as a specific drug target for Wnt/β-catenin signaling appears limited.
Next, I turned my attention to OSTM1, a type I transmembrane protein primarily expressed in osteoclasts. Human mutations in the OSTM1 gene lead to osteopetrosis, a disease of high bone mass. Little is known about the cellular role of OSTM1, with reports suggesting it acts as a cofactor for Cle-7 and as an E3 ubiquitin ligase for the heterotrimeric G-protein Gαi3. Recently, mutations in Wnt/β-catenin pathway components have been shown to generate bone mass defects.

Therefore, I investigated the possible role of OSTM1 in Wnt signal transduction. Overexpression of OSTM1 potentiated Wnt3a-induced β-catenin accumulation and Lef/Tcf-mediated gene transcription. Conversely, knockdown of endogenous OSTM1 by siRNA attenuated signaling. In order to determine if human mutations in OSTM1 had any effect on Wnt/β-catenin signaling, I overexpressed a truncated form of the protein, matching a reported human genetic defect from an osteopetrotic patient. This mutant protein (OSTM1ΔC) inhibited Wnt3a-mediated signaling, consistent with the gene knockdown results. Furthermore, I was able to pinpoint the level of action of OSTM1 through stimulation of the Wnt pathway by several downstream signaling elements. This experiment showed that OSTM1ΔC attenuates Wnt signaling downstream of β-catenin activation, yet upstream of the β-catenin/Lef1 interaction. In fact, through an analysis of β-catenin/Lef1 binding I showed that OSTM1 potentiated, while OSTM1ΔC attenuated, association of these two proteins.

Several important questions are raised by these results. How does OSTM1 regulate binding of β-catenin and Lef1? It will be informative to determine if OSTM1 interacts directly with either protein. Alternatively, OSTM1ΔC may disrupt the intracellular localization of β-catenin or Lef1, rendering them unable to interact.
Immunoprecipitation of OSTM1, followed by an analysis of binding partners will allow further insight into endogenous OSTM1 function.

Do patients with mutations in OSTM1 display Wnt signaling defects in their osteoclasts? Very little is known about the intrinsic role of Wnt signaling in osteoclast function, as the cells are difficult to isolate and manipulate in vitro. Osteoclast-specific activation or disruption of Wnt/β-catenin signaling through the generation of conditional knockouts in mice will go far in answering this question.

What do these results illuminate about the possibility of developing drugs to target mutant OSTM1-mediated osteopetrosis? If, as we propose, Wnt/β-catenin signaling defects are responsible for the osteopetrotic phenotype, drugs which activate the Wnt pathway would be required for treatment. However, systemic pharmacological induction of Wnt signaling is a dangerous proposition and would most likely lead to cancer formation. Alternatively, bone marrow could be isolated from osteopetrotic patients and used for gene therapy, followed by bone marrow transplant. As OSTM1-deficient patients retain the ability to generate osteoclasts (although they are non-functional), specific targeting of these cells with wildtype OSTM1 would likely rescue the osteopetrotic phenotype.

In conclusion, I have identified two novel proteins which regulate Wnt/β-catenin signaling, RGS19 and OSTM1. Each study presented challenges and unexpected results, leading to the formulation of new hypotheses and research directions. It is my hope that the knowledge gained through these investigations will not only enhance our mechanistic understanding of Wnt/β-catenin signal transduction, but will also lead to the generation of targeted drug therapies for cancer and osteopetrosis.
References


129


differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A 95, 3597-602.