Characterization of the Physiological Role of PDZ-RhoGEF in Drosophila and Mice

By

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A thesis submitted in conformity with the requirements For the degree of Doctor of Philosophy Graduate Department of Medical Biophysics University of Toronto

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Biological outputs of insulin/IGF signaling are regulated through essential mediators, such as IRSs, PI3-kinase, and PKB/Akt. These mediators serve critical roles in signal propagation, feedback, and as junctions for crosstalk with other pathways. Abnormal insulin/IGF signaling results in disease, such as obesity, diabetes, and cancer. Given the vital role of this signaling pathway to human health, unraveling its regulatory mechanisms is crucial. Components of this pathway are highly conserved throughout evolution. PTEN, one of the well-defined regulators of this pathway, functions as a lipid phosphatase that negatively regulates insulin/IGF-1 signaling at the PIP3 level, a phosphoinositol that is upregulated by activated PI3-kinase in both Drosophila and mammals. To discover genetic modulators of PTEN in Drosophila, we performed a loss-of-function genetic screen to identify molecules that modify the phenotype elicited by PTEN overexpression in the Drosophila eye. From this screen, we identified a member of the Dbl-family, the guanine nucleotide exchange factor DRhoGEF2, which suppresses the PTEN-overexpression eye phenotype via its effects on dPKB/dAkt activation. By conducting a genetic rescue, we established that PDZ-RhoGEF, a member of the regulator of G-protein signal (RGS)-like domain containing Rho GEFs (RGS-RhoGEFs) subfamily of Dbl-family GEFs, is the
mammalian counterpart of $\textit{DRhoGEF2}$. PDZ-RhoGEF is essential for cell proliferation and survival through ROCK-dependent activation of IRS/PI3-kinase signaling cascade, which has a major impact on adipose tissue homeostasis. Through an integrative approach, we have demonstrated that DRhoGEF2/PDZ-RhoGEF-dependent signaling has tissue-specific effects on insulin/IGF-signaling throughput in both $\textit{Drosophila}$ and mammals. Particularly, we have demonstrated the role played by PDZ-RhoGEF in diet related pathology, provides an alternative therapeutic opportunity in disease intervention.
Acknowledgement

I would like to thank my supervisor, Dr. Tak Mak, who not only gave me the opportunity to pursue my PhD in his laboratory but also gave me the freedom to carry on this project. I also thank the members of my advisory committee, Dr. Sam Benchimol, Dr. Jeremy Squire, and Dr. Jim Woodgett for their support, advises, and patience through the years.

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China: Han You

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Taiwan: Ing-Chu Ho, Men-Shen Chen, Jassy Wang, and An-Li Sui

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signaling. (in submission).
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</thead>
<tbody>
<tr>
<td>7-AAD</td>
<td>7-aminoactinomycin D</td>
</tr>
<tr>
<td>ADSCs</td>
<td>adipose tissue derived stromal cells</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphatic leukemia</td>
</tr>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>AO</td>
<td>acridine orange</td>
</tr>
<tr>
<td>Arf</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>ARM</td>
<td>armadillo</td>
</tr>
<tr>
<td>Asp</td>
<td>asparagine</td>
</tr>
<tr>
<td>ATP-CL</td>
<td>adenosine triphosphate citrate lyase</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl2-antagonist of cell death</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>CA</td>
<td>constitutively active</td>
</tr>
<tr>
<td>CAP</td>
<td>c-Cbl-associated protein</td>
</tr>
<tr>
<td>CCDN1</td>
<td>D1 cyclin gene</td>
</tr>
<tr>
<td>Cdc42</td>
<td>cell division cycle 42</td>
</tr>
<tr>
<td>CdGAP</td>
<td>Cdc42 GTPase-activating protein</td>
</tr>
<tr>
<td>CDM</td>
<td>Ced-5, DocK180, Myoblast city</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>CCAAT/enhancer binding protein a</td>
</tr>
<tr>
<td>Chico</td>
<td><em>Drosophila</em> insulin receptor substrate</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myeloid leukemia</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CR</td>
<td>conserved region</td>
</tr>
<tr>
<td>CR domain</td>
<td>cysteine rich domain</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42 and Rac interacting binding domain</td>
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<tr>
<td>Cta</td>
<td>Concertina</td>
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<tr>
<td>CZH</td>
<td>Ced-5, Dock180, and Myoblast city and Zizimin homology</td>
</tr>
<tr>
<td>Dbl</td>
<td>diffuse B cell lymphoma</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology</td>
</tr>
<tr>
<td>Dia</td>
<td>Diaphanous</td>
</tr>
<tr>
<td>dInR</td>
<td><em>Drosophila</em> insulin receptor</td>
</tr>
<tr>
<td>Dlg</td>
<td>Disc large</td>
</tr>
<tr>
<td>DN</td>
<td>dominant negative</td>
</tr>
<tr>
<td>dPI3-kinase</td>
<td><em>Drosophila</em> phosphoinositol 3' kinase</td>
</tr>
</tbody>
</table>
dPTEN  
Drosophila phosphatase tensin homology

DRok  
Drosophila Rho kinase

dTor  
Drosophila target of rapamycin

EAAT4  
excitatory amino acid transporter 4

ECM  
extracellular matrix

EGF  
epidermal growth factor

ELMO  
engulfment and cell motility

EMS  
Ethylmethanesulfonate

EP  
enhancer-promoter

ERK  
extracellular signal regulated kinase

EYE  
eyeless

F-actin  
filamentous actin

FCS  
fetal calf serum

FFA  
free fatty acid

FIR  
FERM domain including RhoGEF

FIRKO  
fat-specific insulin-receptor knockout

Fog  
folded gastrulation

FOXO  
Forkhead box O transcription factor

Gα12/13  
G protein α-subunit family 12 and 13

GAL4  
yeast transcription factor

GAP  
GTPase activating protein

Gβγ  
G protein βγ-subunit

GDI  
guanine nucleotide dissociation inhibitor

G-domain  
GTP binding domain

GDP  
guanine nucleotide diphosphate

GEF  
guanine nucleotide exchange factor

GFP  
green fluorescent protein

GIT1  
G protein-coupled receptor interacting 1

GLUT4  
glucose transporter 4

GMR  
glass multimer reporter

Grb2  
growth factor receptor bound protein 2

GSK3  
glycogen synthase kinase 3

GTP  
guanosine 5’-triphosphate

GTRAP41  
glutamate transporter EAAT4 associated protein 41

GTRAP48  
glutamate transporter EAAT4 associated protein 48

GTT  
glucose tolerance test

IEG  
immediate early gene
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>Insulin-like growth factor-1 receptor</td>
</tr>
<tr>
<td>IPCs</td>
<td>insulin producing cells</td>
</tr>
<tr>
<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>ITT</td>
<td>insulin tolerance test</td>
</tr>
<tr>
<td>LARG</td>
<td>leukemia-associate RhoGEF</td>
</tr>
<tr>
<td>LH</td>
<td>Lsc homology</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>Lsc</td>
<td>Lbc second cousin</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<tr>
<td>Mbc</td>
<td>myoblast city</td>
</tr>
<tr>
<td>MEFs</td>
<td>mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MF</td>
<td>morphogenetic furrow</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>magnesium ion</td>
</tr>
<tr>
<td>MLC</td>
<td>myosin light chain</td>
</tr>
<tr>
<td>MLL</td>
<td>mixed-lineage leukemia</td>
</tr>
<tr>
<td>MSCs</td>
<td>Melanocyte stem cells</td>
</tr>
<tr>
<td>mTORC</td>
<td>mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PBD</td>
<td>p21-binding domain</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PDK1</td>
<td>phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PDZ</td>
<td>PSD, Dlg, ZO-1</td>
</tr>
<tr>
<td>PDZ-RhoGEF</td>
<td>PDZ domain-containing RhoGEF</td>
</tr>
<tr>
<td>P-element</td>
<td><em>Drosophila</em> transposon</td>
</tr>
<tr>
<td>PGC-1a</td>
<td>proliferators-activated receptor-coactivator</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PHTS</td>
<td>PTEN hamartoma syndrome</td>
</tr>
<tr>
<td>PI</td>
<td>propidium Iodide</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>phosphotidylinositol 3' kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PIX</td>
<td>Pak-interactin exchange factor</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKB/Akt</td>
<td>protein kinase B/mouse leukemia virus AKT8</td>
</tr>
</tbody>
</table>
PKC  protein kinase C
PKN  protein kinase N/protein kinase C-related protein kinase
PMSF phenylmethanesulfonyl fluoride
PPARγ peroxisome proliferators-activated receptor γ
PR  photoreceptor
PRAS40 proline-rich Akt substrate of 40 kDa
PSD postsynaptic-density protein
PTB  phosphotyrosine binding
PTEN phosphatase tensin homology
Rab  Ras-like proteins in brain
Rac 1 Ras related C3 botulinum toxin substrate 1
Ran  Ras-like nuclear
Ras  Ras sarcoma
RBD  Rho binding domain
RGS  regulator of G protein signaling
RGS-RhoGEF regulator of G protein signaling domain-containing RhoGEF
Rheb Ras homolog enrich in brain
Rho  Ras homology
Rho GEF  Rho-subfamily guanine exchange factor
RhoGAP GTPase activating protein for Rho-family GTPases
RhoGEF DH domain guanine nucleotide exchange factor for Rho-family GTPases
ROCK  Rho-associated coiled-coil-containing protein kinase
ROS  reactive oxygen species
R-Ras related Ras viral (r-ras) oncogene homology
RTK receptor tyrosine kinase
SCA1 spinocerebellar ataxia 1
SH2-domain Src-homology 2
SH3  Src-homology 3
SHP  SH2-containing phosphatase-2
SOD-1  superoxide dimuatase 1
Src  sarcomas
STEF  SIF and Tiam 1 like exchange factor
TEP1 Tumor growth factor enhance protein
Tiam 1  T-lymphoma invasion and metastasis 1
TOR  target of rapamycin
TSC1 tuberous sclerosis complex 1, hamartin
TSC2 tuberous sclerosis complex 2, tuberin
UAS  upstream activation sequence
VEGF  vascular endothelial growth factor
VLDL  very-low density lipoprotein
WASP  Wiskott-Aldrich syndrome protein
WAVE  Wiskott-Aldrich syndrome protein-family verprolin-homologous protein
xIRS-1  *Xenopus* insulin receptor substrate-1
ZO-1   Zona occludens-1
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Chapter I

Introduction
**Rho (Ras homology) family of small GTPases**

Members of the Rho family of small GTPases (Rho-family GTPases) function as molecular switches to control a wide range of cellular processes. This family belongs to the Ras superfamily of small GTPases. Rho family proteins not only exhibit high sequence homology but also share similar structural features and biochemical activity with Ras, the founding member of Ras superfamily small GTPases. Like the other members in the Ras superfamily, including Ras (Ras sarcoma), Rab (Ras-like proteins in brain), Ran (Ras-like nuclear protein), and Arf (ADP-ribosylation factor), Rho-family proteins are conserved throughout eukaryotic evolution (1-5).

On the basis of primary amino acid sequence identity, structural motifs and biological function, members of the Rho family can be divided into eight major subfamilies including the classical members Rho (RhoA, B, C), Rac (Rac1, Rac2, Rac3, and RhoG), Cdc42 (Cdc42/G25K, TC10, TCL), and RhoD/RhoF as well as atypical Rho GTPases Rnd (Rnd1, Rnd2, and RhoE/Rnd3), RhoBTB (1 and 2), RhoV/RhoU, and RhoH (Figure I-1).

Among all Rho subfamily proteins, Cdc42, Rac, and Rho are not only the most studied from yeast to human but also the most highly conserved throughout evolution. Interestingly, recent analysis has demonstrated that all Rho family small GTPases originated from an ancestral molecule, Rac, suggesting that Cdc42 and Rho have evolved from a Rac duplication associated with early specialization in functions, and implicating Cdc42 in the control of cell polarity and Rho in cytokinesis (5-7). The fundamental cellular processes regulated by Rho-family proteins
Figure I-1. The human Rho-family GTPases.
An unrooted phylogentic tree is based on ClustlW alignment of the amino-acid sequence of 20 Rho family GTPases. This tree presents the relationship between different family members. Rho family GTPases includes 8 subfamilies: 4 classical Rho GTPases (Rho, Rac, Cdc42, RhoF/RhoD) and 4 atypical Rho GTPases (RhoB, RhoV/RhoU, RhoH, and Rnd). Rac and Rho subfamily proteins exhibit highest sequence similarity. Other subfamilies display less sequence similarity. Classical members Rho GTPases cycle between an inactive GDP-bound form and an active GTP-bound form by Rho GEFs and Rho GAPs. Whereas, atypical Rho GTPases predominantly exist in an active GTP-bound form. Thus, they are not regulated by Rho GEFs and Rho GAPs, instead, they are regulated at transcriptional and post-translational levels. (adapted from ref 24)
are also common to all eukaryotes, implying that other components of Rho signaling pathways are also evolutionarily conserved. Indeed, accumulating evidence has shown the presence of regulators and effectors of Rho-family GTPases in all eukaryotic genomes (8-10).

**Regulation of GTPase activity**

Rho family small GTPase activity is determined by the bound nucleotide: the GDP-bound inactive state and the GTP-bound active state. In contrast to the GDP-bound molecule, the GTP-bound form actively transduces signals by interacting with downstream effectors (11, 12). Cycling between GTP-bound and GDP-bound states is critical for Rho signaling mediated biological functions. These molecules are tightly controlled by two distinct families of proteins: Rho-family specific guanine nucleotide exchange factors (Rho GEFs) that facilitate GTP nucleotide binding (13, 14), and Rho-family specific GTPase-activating proteins (Rho GAPs) that stimulate GTP hydrolysis (15, 16) (Figure I-2). In addition to nucleotide binding, guanine nucleotide dissociation inhibitors (GDIs) and C-terminal lipid modification are essential for directing the subcellular localization of Rho GTPases (17-19), which is important for its biological functions.

**Rho-family GTPases**

Three members of Rho-family GTPases, Cdc42, Rac, and Rho are ubiquitously expressed. Altered Rho GTPase signaling has been associated with various developmental defects and the etiology of many human diseases, suggesting that these small GTPases play
Figure I-2. The GTPase cycle

The GTPase cycle includes three conformational states of the protein. GDP is released from an inactive form of GDP-bound GTPase to yield a transient nucleotide-free or ‘empty’ state of the protein. Subsequently, GTP enters the empty guanine nucleotide binding site and the GTPase takes on an ‘active’ conformation, which reverts to the GDP-bound inactive state when GTP is hydrolyzed. The GTPase cycle is controlled by two classes of regulatory proteins: guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs stabilize nucleotide-free GTPase for GTP binding. GAPs stimulate intrinsic hydrolytic capacity of GTPases to promote GDP-bound form and terminate the signaling. An additional regulator for Rho and Rac family proteins is guanine nucleotide dissociation inhibitors (GDI). GDIs inhibit nucleotide exchange by masking the lipid modification and sequestering Rho and Rac GTPases in the cytosol. (modified from ref 192)
fundamental roles in the maintenance of cellular homeostasis. Rho family proteins control numerous cellular processes ranging from the regulation of the actin-based cytoskeleton, cell morphology (9), motility (20, 21), vesicle transport (22), nuclear gene expression (23), and cell growth to development and differentiation (8). Importantly, global and tissue-specific loss-of-function studies of Rho GTPases have further supported these findings (24).

Cdc42-subfamily GTPases

Cdc42 regulates filopodia formation at the leading edge of migrating neuronal cells by binding to Wiskott-Aldrich syndrome protein (WASP) or through binding to the insulin receptor tyrosine kinase substrate p53 (IRSp53). In addition to WASP and IRSp53, p21-activated kinase (PAK) and mammalian diaphanous 2 (mDia2) are also involved in Cdc42-mediated filopodia formation (25, 26). Mice lacking Cdc42 are embryonic lethal and Cdc42-null cells show no or reduced filopodia (27-30). Analysis of tissue-specific deletion of Cdc42 has revealed its role in mediating filopodia formation in a cell- or tissue-specific manner. Furthermore, loss of Cdc42 affects filopodia-dependent cellular processes, such as migration, axon extension or growth, cell polarity, migratory polarity, cell fate determination in hematopoietic lineages and brain (24). Instead of affecting axon extension, deletion of Cdc42 in brain leads to the loss of filopodia formation and reduced axon number (31-36). These findings support the roles of Cdc42 in the regulation of cell polarity, migratory polarity, and cell fate specification are conserved from budding yeast Saccharomyces cerevisiae to mammals (24, 37).
Rac subfamily GTPases

Lamellapodium, a dendritic network of branched actin filaments is formed at the leading edge of the cell when Rac proteins (Rac1, 2, 3 and RhoG) are activated (38). Rac activates actin polymerization during lamellapodium formation directly through a Wiskott-Aldrich syndrome protein (WASP) family verprolin-homologous protein (WAVE) complex or indirectly through IRSp53, which activates actin-nucleating complex Arp2/3 (39, 40). In addition, PAKs are activated by Rac in regulating actin polymerization during lamellapodium formation (26, 41). Rac proteins have different expression patterns: Rac1 and RhoG are widely expressed, whereas Rac2 is restricted to cells of hematopoietic origin and Rac3 is only found in brain (42-46). Importantly, Rac1-null mice are embryonic lethal due to defects in germ layer formation (47), suggesting that Rac1-mediated lamellipodia is required for embryogenesis. Rac2-, Rac3-, and RhoG-deficiency have no effects on embryo development. Studies from tissue-specific deletion of these molecules also demonstrate that Rac-family members display non-redundant functions in mediating lamellapodia-related functions in hematopoietic cells, endothelial cells, and Schwann cells (48-55). Significantly, Rac1 and Rac2 are involved in nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase activation in cardiomyocytes and leukocytes, respectively (56-58). Interestingly, higher reactive oxygen species (ROS) levels in Rac1-null embryonic fibroblasts are a consequence of upregulation of Rac3, which leads to DNA damage and a p53-dependent senescence (59). Together, this suggests that Rac family members
regulate ROS production in a tissue- or cell-specific manner.

**Rho subfamily GTPases**

The Rho subfamily of GTPases includes RhoA, B, and C. These proteins are highly homologous in protein sequence. When they are overexpressed as activated mutants, contribute to myosin-dependent contractility of actin filaments leading to the formation of actin stress fibers and focal adhesions, mediated through diaphanous-related formins (DRFs, including mDias and Rho-associated coil-coiled containing protein kinases (ROCKs) (60, 61). Despite their high homology in protein sequence, studies using dominant-negative forms of RhoA, RhoB, and RhoC have clearly shown that these three small GTPases play distinguishable roles in cells (62). Such non-redundant roles for RhoA, RhoB, and RhoC may be due to their distinct subcellular localizations and binding to specific regulator and effector. Indeed, unique functions have been demonstrated in RhoB-null and RhoC-null mice (63, 64).

RhoB is found predominantly localized in late endosomes/lysosomes (65). Analysis of RhoB-null cells further supports its role in regulation of vesicle trafficking. Importantly, RhoB regulates trafficking of vesicles and therefore participates in signal transduction for cell survival and proliferation (66). Interestingly, RhoB-null mice are susceptible to carcinogen-induced skin tumor formation (67). Moreover, neoplastically transformed RhoB-null MEFs have defects in adhesion and spreading, and these cells are more sensitive to transforming growth factor β (TGF-β)-induced changes in actin cytoskeleton and cell proliferation (63). In addition, RhoB is
involved in stage-specific survival of endothelial cells by controlling PKB/Akt trafficking during vascular development (68). These findings suggest that RhoB has a major role in negatively regulating integrin and growth factor signals that are required for neoplastic transformation, which is in consistent with its inhibitory effect on growth, metastasis, and invasion (69-71). In contrast, RhoC promotes cell growth, metastasis, and invasion when overexpressed (72-75). RhoC-null mice exhibited decreased tumor cell motility and metastatic cell survival, which leads to a drastic inhibition of metastasis (64). In line with this, microRNA \textit{mir-10b}, that is found in metastatic breast cancer, stimulates invasion \textit{in vitro} and metastasis \textit{in vivo} through indirectly upregulating RhoC expression (76).

Although RhoA-knockout mice are not yet available, the role of RhoA in neuritogenesis has been suggested. RhoA activation is responsible for LPA-mediated neurite retraction and counterbalances NGF-induced neurite outgrowth (77-81). Furthermore, such effect is confirmed when RhoA activity is inhibited by C3 exoenzyme or reduced by Rho GAP (82-84). In contrast to Rac and Cdc42, RhoA activation has been linked to neurite retraction, growth cone collapse, and cell rounding when the molecule is overexpressed or is activated by various extracellular signals (78, 85-88). RhoA stimulation of myosin II activity in neurons may play an essential role in neurite or axon retraction (89, 90). Importantly, enhanced RhoA activity has been associated with central nerve system (CNS) or spinal cord injury (91) and inhibition of RhoA/ROCK signaling promotes nerve for regeneration (92-95). Together, proper control or counterbalance of
RhoA activity is essential for neuronal homeostasis.

In addition to its effect on the actin cytoskeleton, RhoA regulates the expression of genes associated with cell cycle progression and cell fate determination. Control of actin cytoskeleton-dependent gene expression is established by Rho-family GTPases via serum response factor (SRF), a MADS-box transcription factor (96-98). It has been shown that SRF activity is dependent on actin treadmilling, the head to tail polymerization of actin microfilaments (99-101), and this regulates the subcellular localization and activity of MAL, a G-actin-binding SRF coactivator (102-105). Importantly, SRF-mediated gene expression is enhanced during the mitogen-induced G₀-G₁ transition and is required for PI3-kinase-regulated cell proliferation (106-108), suggesting that SRF/SRE-dependent gene expression is essential for RhoA-mediated cell cycle progression and cell proliferation.

RhoA also regulates cell cycle progression through the actin-independent transcriptional regulation of cell cycle mediators. Cyclin D1 is a sensor and integrator for extracellular signal-mediated G₁/S phase cell cycle transition (109). Its expression increases as cells enter the cell cycle and decreases in the S phase. Moreover, the levels of cyclin D1 is crucial for cell proliferation and tumorigenesis (110, 111). RhoA-induced cyclin D1 expression is required for fibronectin- and Ras-mediated cell cycle progression as well as Ras-dependent and independent cell proliferation (112-115). Cyclin dependent kinase inhibitor (CDKI) p21\(^{\text{Cip1/WAF1}}\), a p53-inducible protein, is a critical regulator of the cell cycle and cell survival (116-120). It has been
shown that activated RhoA represses Sp1-mediated p21<sub>CIP1/WAF1</sub> expression (122). Suppression of p21<sub>CIP1/WAF1</sub> by activated RhoA is required for Ras-dependent S phase entry (114, 121). In addition, activated RhoA suppresses the level of p27<sub>KIP1</sub> possibly through a post-translational mechanism (115, 123, 124). Moreover, RhoA activation enhances cyclin D1 activity and is essential for promoting cell proliferation and cellular transformation of p53-null fibroblasts (125, 126). These findings indicate that activated RhoA signaling pathway has transformation capacity via its role in promoting cell cycle progression and this is distinct from RhoC’s role in enhancing tumor cell motility and invasion. Significantly, elevated expression and activity of RhoA are found in various cancers, including melanoma, lung, colorectal, ovarian, liver, prostate, neuroblastoma, acute myeloid leukemia (AML), and sarcoma (127).

Prior to differentiation, mesenchymal progenitor cells commit to different lineages, including the adipogenic, myogenic, and osteogenic lineages. It has been shown that cell shape change is evident during differentiation, especially noticeable when mesenchymal progenitor cells differentiate into adipocytes, osteoblasts, and myoblasts (128-131). Moreover, it has been shown that adhesion molecule-mediated cell shape change affects the differentiation of pre-committed mesenchymal lineages (132-136). These findings suggest that actin cytoskeleton modeling also plays a role in mesenchymal cell differentiation. The RhoA GTPase is involved in regulating actin cytoskeleton and cell shape change in Drosophila and mammals (137-141). McBeath and colleagues have shown that RhoA-mediated cell shape change plays a central role
in directing the lineage commitment of human mesenchymal stem cells by generating actin-myosin-cytoskeletal tension via activated Rho-kinase(s) (142). Interestingly, a non-actin-centric role of RhoA in mouse adipogenesis-myogenesis cell fate determination also has been proposed (143). These studies have provided new a perspective on RhoA’s function.

**Effectors of Rho GTPases**

In their activated state, Rho-family GTPases interact with their downstream effectors to engage specific signaling cascades (7, 12). Over 70 potential effectors have been identified for Rho-family GTPases and most of these effector molecules are either serine/threonine kinases or scaffold proteins (12, 144). From a structural perspective, effectors bind to Rho-family proteins through the ‘effector region’ within the switch I and II region that forms the major docking/recognition sites (145). The amino acids in the effector region in interaction with each effectors are distinct (146), suggesting that these amino acids play a major role in determining effector specificity. Other sequences outside of the docking/recognition sites of Rho GTPases also has been suggested to participate in effector-mediated functions (146). The best-characterized effectors of Rac1 and Cdc42 are the serine/threonine kinases, p21-activated kinases (PAKs) and ROCKs (ROCKI/β and ROCKII/β) for Rho. Both PAKs and ROCKs are highly conserved in all eukaryotic genomes (147, 148). Rho-family GTPases all activate effectors by inducing a conformational change that removes auto-inhibition (149-151). In addition, the subcellular localization of the effectors is tightly associated with their activation by binding to
specific Rho GTPases. For instance, RhoA directs ROCKs to the cleavage furrow during cytokinesis (152), and RhoE co-localizes with ROCKI/β on trans-Golgi to block ROCKI/β activation resulting in disassembly of stress fibers (153). Moreover, PAK4 is translocated to Golgi apparatus as a result of their binding to Cdc42 (154, 155). Therefore, Rho-family GTPases are essential for enhancing activity and/or tethering effectors to specific sites or compartments where the substrates locate.

**p21 activated kinases (PAKs)**

PAKs are a highly conserved family of proteins with six human members. The six currently known mammalian PAK family members are divided into two groups, group I (PAK1-3 or PKAα, β, γ) and group II (PAK4-6) based on sequence similarity. It has been shown that Rac preferentially interacts with group I PAKs, whereas Cdc42 binds to both groups of PAKs, leading to the regulation of actin cytoskeleton remodeling (156). These kinases contain a Cdc42 and Rac-interacting binding (CRIB) domain within their N-terminal p21-binding domain (PBD) and a C-terminal kinase domain. Other domains, such as auto-inhibitory and PAK-interacting exchange (PIX) factor binding domains, are only found in the group I PAKs but not group II PAKs. Therefore, binding to Cdc42 does not affect kinase activity of group II PAKs (157, 158). The group I PAKs exist as inactive homodimers through interaction between their kinase domains (159). GTP-bound Rac or Cdc42 activates PAKs (group I) through direct binding at PBD, which releases its auto-inhibition and dimerization (159-161). Nonetheless, both groups
are important in cell motility and survival. Subcellular localization of PAK1 at cell membrane, pinocytic vesicle, and cortical actin structures is critical for its activation by Rac and Cdc42 (162, 163). Furthermore, several studies have shown that the effects on Rac1/Cdc42-dependent PAK activation and subcellular localization are directed by the complex of G protein-coupled receptor kinase interacting 1 (ArfGAP/GIT1), a GAP of Arf, and p21-activated kinase interacting exchange factor α (α-PIX), a Rho GEF for Rac/Cdc42 (164-166). Collectively, these findings suggest that PAK activation and their functions are linked to a specific member of Rho-family GTPases and the upstream regulators.

Through various downstream substrates, including paxillin, filamin A, LIM-kinases (LIMKs), and stathmin (167), activated PAKs are involved in reducing stress fiber formation (168), and cell migration through regulation of actin and microtubule dynamics (169-172). In addition to their role in actin remodeling, PAKs are also involved in regulating transcription and translation, cell cycle progression, cell proliferation, and apoptosis (157). A wealth of evidence has shown that altered expression or activity of PAKs is found in variety of human cancers, including breast, ovary, colorectal, thyroid, and pancreatic cancers (173). A hyperactivated PAK1 mouse model has shown that overexpression of catalytically activated PAK1 is sufficient to drive mammary tumor development (174).

It has been suggested that PAK activity is related to processes required for neuronal development, including neuronal polarity, neurite outgrowth and axonal guidance. Knockout
mouse models show that among the PAK family, PAK3 and PAK4 are required for neuronal development (175, 176). Indeed, mutations that may affect PAK activity or expression are associated with several neurological disorders, including Alzheimer and Parkinson diseases, and X-linked mental retardation (177-180). Together, p21-activated kinases play a central role in regulating Cdc42 or Rac mediated cellular processes, such as actin remodeling and cell proliferation. Importantly, altered PAK activity or expression leads to disease development, including cancers and neurological disorders, analogously to deregulated Rac or Cdc42 activity.

**ROCKs**

ROCKs (ROCKI/β and ROCKII/α) are required for RhoA-mediated formation of stress fibers and focal adhesions via their effects on the phosphorylation of myosin light chain (MLC) (61, 181, 182). In general, these serine/threonine kinases (~160 kDa) consist of an N-terminal kinase domain followed by a coiled-coil-containing region, a PH domain and a cysteine-rich domain (CRD) at the C-terminus (183). Within the coiled-coil-forming domain, there is a Rho-binding domain (RBD) that interacts with GTP-bound RhoA (184, 185). It has been shown that Rho-kinase activity is negatively regulated through an auto-inhibition that is formed by the intramolecular interaction between C-terminal region and N-terminal kinase domain of ROCKs (151). Upon binding to GTP-bound RhoA, autoinhibition is released, and ROCKs are activated for substrate phosphorylation (151). ROCKI/β and ROCKII/α transcripts are ubiquitously expressed (183). ROCKII/α transcripts are highly abundant in brain and skeletal muscle,
whereas ROCKI/β transcripts are barely detected in those tissues (181). This suggests that ROCKI/β and ROCKII/α may have tissue-specific functions.

It is believed that both ROCKs recognize the same substrate sequence due to their highly homologous kinase domain (183). Activated ROCKs phosphorylate several substrates at consensus amino acid sequences R/KXS/T or R/KXXKS/T (where R is arginine, K is lysine, X is any amino acid, S is serine, and T is threonine), leading to various cellular responses (186, 187). Most of these substrates are involved in RhoA/ROCK mediated actin cytoskeleton remodeling and thus regulate cell migration and invasion, adhesion/differentiation, phagocytosis, neurite retraction, as well as cytokinesis and mitosis (148). As mentioned in the previous section, an increase in the protein level or activity of RhoA leads to cell transformation and tumorigenesis and ROCK activity is required for this transformation (188-192).

The actin-centric role of ROCKs is to regulate morphological change by promoting anchorage-independent cell growth of transformed cells. ROCKs have also been implicated in cell shape-mediated mesenchymal stem cell lineage commitment downstream of RhoA (142). Interestingly, a non-actin-related role of ROCKs in modulating insulin signaling has been revealed. Farah and colleagues first demonstrated that the *Xenopus* homolog of RhoA-associated coil-coiled protein kinase (xROCKα) is a binding partner of *Xenopus* insulin receptor substrate 1 (xIRS-1) and binding of these two molecules leads to inhibition of insulin signaling in *Xenopus* oocytes (193). Accumulating evidence in mammals has shown that ROCKs not only interact
with IRS-1 but also phosphorylate IRS-1 at serine residues S612 and S632/635 in mediating insulin-dependent glucose transport and adipocyte differentiation (143, 194-197). Importantly, mice lacking ROCK1/β causes systemic insulin resistance by impairing insulin signaling in skeletal muscle due to reduction of IRS-1-associated activation of PI3-kinase signaling cascade (198). Thus, this finding has provided new perspectives of RhoA signaling in modulating whole body glucose homeostasis.

**Regulators of Rho-family GTPases**

GEFs for Rho-family proteins are classified on the basis of both sequence similarity and substrate specificity. The first mammalian Rho GEF, Dbl, was identified as a transforming gene from Diffuse-B-cell-lymphoma cells (199). Dbl contains an approximately 240 amino acid long region known as the Dbl homology (DH) domain. The DH domain is homologous to a region in Saccharomyces cerevisiae Cdc24 known to regulate Cdc42 activity in this organism (200-203). Dbl was also shown to function as a GEF for human Cdc42 and since then, 69 distinct members of the Dbl family have been identified in humans. Rho GEFs are conserved throughout eukaryotic evolution (204, 205). Rho GEFs are divided into two major subfamilies based on the presence of a catalytic domain for guanine nucleotide exchange: the DH-domain containing Rho GEFs (referred to as DH-Rho GEFs) and newly identified atypical Rho GEFs which lack DH domains. These atypical RhoGEFs without DH domain are known as CZH proteins. CZH proteins are divided into two groups, the CDM (Ced-5, Dock180, and Myoblastcity) proteins and
the Zizimin homology proteins (13, 206, 207). The DH-Rho GEF family members activate Cdc42, Rac, and Rho subfamilies of small GTPases, whereas, CZH proteins selectively activate Rac and Cdc42 subfamilies. Therefore, DH-Rho GEFs are considered as the universal GEFs for Rho-family GTPases in eukaryotes. The DH domain of Rho GEFs forms a catalytic module with the adjacent, C-terminal 100 residue pleckstrin homology (PH) domain. The PH domain of catalytic module possesses phosphoinositol lipid-binding activity, which plays a role in targeting Rho GEFs to the cell membrane. Furthermore, the PH domain is required for full catalytic activity of DH domain in regulating GDP/GTP exchange of Rho GTPases. Other functional domains, including domains that promote oligomerization, protein-protein interactions or membrane interactions, as well as regions with unknown functions, flank the DH-PH catalytic module of Rho GEFs. These domains are believed to be essential for allowing Rho GEFs to receive upstream signals through interaction with upstream receptors and signaling molecules. Therefore, Rho GEFs functions as signaling integrators in resulting the activation of Rho GTPases (13, 208-212).

**Rho GEFs and Nucleotide binding**

Although the catalytic domains of the different classes of GEFs do not share sequence homology and are not structurally related (213, 214), they share a common mechanism of nucleotide exchange. The DH domain of Rho GEFs is arranged as α-helices and contains three highly conserved regions (CR1-3). Guanine nucleotide exchange is mediated by interaction
between conserved regions of the Rho GEFs and the switch regions of Rho-family proteins. This interaction results in a structural change in the P loop (nucleotide binding site) of the Rho family small GTPases and this is believed to facilitate the release of GDP as well as to stabilize the nucleotide-free (empty state) intermediate of the GTPase, that is subsequently bound with GTP (206, 215-217) (Figure I-2).

**Regulation of DH-Rho GEFs**

Altered expression or activity of DH-Rho GEFs is associated with defects in development as well as etiology of diseases (15, 216). Apart from their catalytic domain, the DH-Rho GEFs also contain a diverse set of functional domains, including Src homology 2 and 3 (SH2 and SH3) domains, the PDZ (postsynaptic-density protein (PSD) 95 kD, Disc large, and Zona occludens-1) domain, serine/threonine kinase domains, or additional PH domains. These domains are believed to be essential in allowing DH-Rho GEFs to receive signals through interaction with upstream receptors and other signaling molecules resulting in specific activation of Rho GTPases and regulation of their catalytic activity. Indeed, DH-Rho GEFs have been implicated in the regulation of Rho-family GTPase signaling downstream of multiple cell surface receptors such as receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), adhesion molecules, as well as plexin receptors (209-212, 218-220). Although no universal regulatory mechanisms have been defined, in general, DH-Rho GEFs are regulated by various mechanisms including protein-protein interaction, lipid binding, and posttranslational modification (206, 216). These
mechanisms may affect GEF activities fundamentally by releasing intra-molecular inhibitions and thus increasing accessibility to Rho-family small GTPases and/or by mediating signaling fidelity and specificity of the Rho-family small GTPases (13).

To date, over 70 DH-Rho GEFs, 60 Rho GAPs, and 70 Rho effectors have been identified in higher eukaryotes (10, 12, 13, 16, 144). This diversity has made it difficult to elucidate the complex in vivo relationships in the signaling pathways in which these molecules act. In addition, crosstalk with other Ras-superfamily small GTPases has further complicated the understanding of the biological and biochemical outputs of Rho-family GTPases (221). Considering the complexity of regulation of Rho-family GTPases by DH-Rho GEFs and the signaling outputs through downstream effectors, the biological functions of these molecules must be regulated spatio-temporally in specific cells or tissues. Recently, a subfamily of DH domain containing proteins was found to link various signals from GPCRs, insulin-like growth factor 1 (IGF-1) receptor (IGF-1R), and plexin B specifically to RhoA GTPase-activation (222-225). In addition to their catalytic DH-PH domain, the members of this subfamily of GEFs contain a unique regulator of G-protein signaling (RGS)-like domain N-terminal to the DH domain. The RGS domain, also known as an RGS-box domain, is an approximately 120 amino acid long module that defines members of the superfamily of RGS proteins and acts as Gα-subunit-specific GAPs for heterotrimeric G proteins (226, 227). The interaction between RGS proteins and Gα-subunit attenuates G-protein mediated responses in all eukaryotes from yeast to
mammals (228-231). These members are classified as RGS-RhoGEFs. As for RGS-RhoGEF, the RGS-like domain, also known as Lsc homology (LH) domain, was initially identified by its sequence homology to Lbc second cousin (Lsc) (232). Subsequently, similar regions were also found in p115 RhoGEF (human ortholog of Lsc), Leukemia-associated RhoGEF (LARG), PDZ-domain containing Rho family GEF (PDZ-RhoGEF), as well as *Drosophila* Rho family GEF 2 (DRhoGEF2) (222, 233-235) (Figure I-3). The RGS-like domain of RGS-RhoGEFs specifically interacts with Gα12 and Gα13 to accelerate their intrinsic GAP activity, leading to inactivation of Gα12 and Gα13 mediated signaling cascades (222, 236-240). Simultaneously, such interaction may affect nucleotide exchange activity of these RGS-RhoGEFs, thus providing a direct link between heterotrimeric G proteins and RhoA (223, 237). Through sequence analysis, these proteins not only share similar domain structures but also display sequence homology (Figure I-3). Therefore, the RGS-RhoGEF subfamily includes p115RhoGEF, LARG), PDZ-RhoGEF, and their *Drosophila* ortholog, DRhoGEF2. Interestingly, a PDZ domain is found only at N-terminus of DRhoGEF2, LARG, and PDZ-RhoGEF (Figure I-3). Interaction between the RGS-like domain of p115 RhoGEF, LARG and PDZ-RhoGEF and the Gα12/13 has been demonstrated (222, 236-241). Although there is no evidence that indicates a direct interaction between DRhoGEF2 and *Drosophila* ortholog of Gα proteins, Concertina (Cta), genetic studies have shown that DRhoGEF2 is required for Folded gastrulation (Fog)/Cta-mediated embryogenesis (234, 235), pointing to a similar mechanism for the Gα-coupled receptor mediated signaling
Figure I-3. Functional domains and sequence homology between *Drosophila* and mammalian members of RGS-RhoGEF subfamily.

Schematic representation of primary protein structure between DRhoGEF2 and mammalian orthologs Members of RGS-RhoGEFs: PDZ-RhoGEF, PDZ-RhoGEF^Δ8^ (an alternative spliced variant lacking exon 8), Leukemia-associated RhoGEF (LARG), and p115RhoGEF (human ortholog of *Lbc* second cousin (Lsc)). PDZ domain: postsynaptic-density protein (PSD) 95kD-Disc large-Zona occludens-1 domain, PRD: proline-rich domain, RGS domain: regulator of G protein signaling domain, DH domain: Dbl homology, PH domain: pleckstn homology.
pathways in *Drosophila* and mammals (Figure 1-4). For the purpose of this thesis, current knowledge of PDZ-RhoGEF and its mammalian homologues, p115 RhoGEF and LARG, will be summarized in the following sections

**p115RhoGEF/Lsc**

Through its RGS-like domain, p115RhoGEF/Lsc interacts with Gα13, which increases its catalytic activity towards RhoA (238). Gα13 activates RhoA-induced SRE-dependent geneexpression through proline-rich tyrosine kinase 2 (Pyk2), which is inhibited by overexpression of the RGS-like domain of p115RhoGEF/Lsc (242). This suggests that the RGS-like domain is a negative regulator of Gα13-coupled receptor signaling. As with the PH domain, the RGS-like domain is required for plasma membrane recruitment of p115RhoGEF/Lsc, and both domains contribute to receptor binding (243). In addition, the C-terminal region of p115RhoGEF/Lsc is required for homo-dimerization and regulates the nucleotide exchange activity of p115RhoGEF/Lsc. Deletion of this region results in enhanced GEF activity *in vivo* (244). The expression of p115RhoGEF/Lsc is mainly restricted to the hematopoietic system, including the thymus, spleen, and lymph nodes (245). Mice lacking p115RhoGEF/Lsc are viable and healthy (245). Consistent with its limited expression pattern, analysis of p115RhoGEF/Lsc-null mice has demonstrated that p115RhoGEF/Lsc is required for marginal zone B cells and leukocyte homeostasis (245, 246).

**LARG**
Figure I-4. Conserved Gα-coupled receptor-mediated signaling pathway.

G-protein-coupled receptors (GPCRs) represent a large family of seven transmembrane receptors. Upon ligand binding (e.g. LPA), GPCRs catalyze the exchange of GDP for GTP on Gα subunits (Gs, Gi, Gq, and Gα12/13) and release of Gβγ subunits from the heterotrimeric G protein. Both GTP-Gα subunit and free Gβγ subunit interact with diverse downstream effectors. GTP-Gα12/13 interacts with a subfamily of Rho GEFs, RGS-RhoGEFs, including p115RhoGEF, PDZ-RhoGEF, and leukemia-associated Rho GEF (LARG) through RGS-like domain of these Rho GEFs. RGS-RhoGEFs act as a GAP to accelerated intrinsic GTPase activity of GTP-Gα12/13. Binding to GTP-Gα12/13 may enhance catalytic activity of RGS-RhoGEFs for GDP/GTP exchange for RhoA GTPase in modulating various cellular processes. Drosophila Gα-protein, Concertina (Gta) and DRhoGEF2 downstream of folded gastrulation (Fog) are closely related to GTP-Gα12/13 and RGS-RhoGEFs, respectively, pointing to a conserved mechanism of Gα-coupled receptor-mediated signaling pathway in Drosophila.
LARG is encoded by the *ARHGEF12* locus located on human chromosome 11q23.3 (mouse chromosome 9) in a region telomeric to myeloid-lymphoid leukemia (MLL). LARG was first identified as a fusion partner of MLL in a patient with primary acute myeloid leukemia (AML) (233). In addition to the functional domains found in p115RhoGEF/Lsc, LARG contains an additional PDZ domain at its N-terminus (233). Similar to p115RhoGEF/Lsc, LARG also acts downstream of Gα12/13-coupled receptors leading to RhoA activation through direct interaction with Gα12 and Gα13 via its RGS-like domain (241). Overexpression of the RGS-like domain of LARG alone diminishes Gα12 and Gα13 induced SRE-dependent gene expression (241), suggesting that LARG also functions as a negative regulator of the Gα12/13-coupled receptor mediated signaling cascade. Like p115RhoGEF/Lsc, the catalytic activity of LARG is regulated through its C-terminal region that is involved in oligomerization, and is inhibitory for RhoA activation and SRE-dependent gene expression (244). Moreover, LARG mediated RhoA activation leads to activation of focal adhesion kinase (FAK), which in turn forms a positive feedback loop for sustaining RhoA activation by tyrosine phosphorylation of LARG (247). LARG is also known to interact with the IGF-1R and as well as plexin B, the semaphorin receptor; through its PDZ domain, leading to RhoA activation and thereby regulates stress fiber formation and growth cone morphology (248, 249).

LARG is ubiquitously expressed in human and mouse (233, 248, 250), and its expression is upregulated by the vasoconstrictor angiotensin II in smooth muscle cells (251). Recently,
Wirth and colleagues demonstrated that Gα12/13 and its downstream effector, LARG, are required for salt-induced hypertension in vascular smooth muscle (252). In the murine CNS and peripheral nervous systems (and PNS), LARG expression was found in the soma of neurons where it co-localized with Gα12 (250), suggesting that LARG may be involved in the regulation of neuronal morphogenesis downstream of GPCRs. Significantly, from linkage analysis, a locus potentially associated with obesity and young-onset diabetes was mapped to chromosome 11q23-24 in the region where LARG is located (233, 253). A functional Tyr1306Cys variant in the C-terminus of LARG is associated with increased insulin sensitivity, suggesting that LARG, through its activation of RhoA, may affect insulin sensitivity in non-diabetic population as has been shown in Pima Indians (254).

**PDZ-RhoGEF**

*ARHGEF11*, a locus on human chromosome 1q21 (mouse chromosome 3) encodes the Rho guanine nucleotide exchange factor, PDZ-RhoGEF with two transcript variants, isoform 1: an alternative spliced variant lacking exon 8 (PDZ-RhoGEFΔ8) and isoform 2: full length variant (PDZ-RhoGEF) (222). The mouse has only one known transcript that is highly homologous to human isoform 2. As a member of the RGS-RhoGEF subfamily, PDZ-RhoGEF shares a similar domain architecture with LARG, including a PDZ domain, an RGS-like domain, a catalytic DH-PH domain, and an additional proline-rich motif at the C-terminus of its PDZ domain (222, 225) (Figure I-4). PDZ-RhoGEF physically interacts with Gα12/13 through its RGS-like domain (222,
224), and this leads to RhoA activation, an event essential for neurite retraction and growth cone collapse (224, 255, 256). In contrast to p115RhoGEF/Lsc, PDZ-RhoGEF interacts with G\(\alpha_{13}\) but does not act as a GAP in stimulating the intrinsic hydrolytic capacity of G\(\alpha_{13}\) (236). Furthermore, through its PDZ domain, PDZ-RhoGEF forms a complex with LPA receptors and semaphorin 4D-activated plexin B1. Formation of this complex leads to RhoA activation and stress fiber formation, axon guidance, growth cone collapse, and cell migration (249, 257, 258).

Moreover, PDZ-RhoGEF is also involved in angiotensin II and air pollutant associated vascular hypertension through activation of the RhoA/ROCK signaling cascade (259, 260). Taken together, as a signaling integrator, PDZ-RhoGEF couples various extracellular signals to RhoA activation, which is essential for actin cytoskeleton rearrangement and actin cytoskeleton-related processes.

Like LARG, PDZ-RhoGEF mediated RhoA activation is also regulated through a positive feedback loop involving GPCR-activated FAK (247). Recently, FAK in concert with PDZ-RhoGEF was shown to activate RhoA/ROCK\(\alpha\)-dependent adhesion movement and trailing-edge retraction in response to LPA (261). These findings imply that the receptor-activated tyrosine kinase FAK may have a role in modulating the nucleotide exchange activity of PDZ-RhoGEF. Furthermore, Zheng and colleagues have shown that the RGS-like domain and a unique sequence motif upstream of the DH domain of PDZ-RhoGEF cooperatively auto-inhibits the ability of the DH domain to bind to nucleotide free RhoA (262), suggesting that the catalytic
activity of PDZ-RhoGEF is regulated through intra-molecular auto-inhibition. Moreover, homo- and hetero-oligomerization via the unique C-terminal regions of PDZ-RhoGEF, LARG, and p115RhoGEF/Lsc, results in an inhibitory effect on their catalytic activity and transformation potential (244), indicating that the GEF activity of PDZ-RhoGEF is regulated through an inter- and/or intra-molecular interactions. Most of the GEFs regulate multiple small GTPases; RhoA specific GEF activity is one of the distinct features of RGS-RhoGEFs. This substrate specificity of PDZ-RhoGEF is likely determined by the interaction between both the DH and PH domains of this GEF and the switch regions of RhoA (263-266).

It has been shown that PDZ-RhoGEF is ubiquitously expressed with highly abundant expression in the brain (222). PDZ-RhoGEF expression is detected in neuropil (dendrites, unmyelinated and myelinated axons) of hippocampal neurons, in cell bodies and dendrites of Purkinje cells, as well as in nociceptive afferent neurons in the peripheral nervous system (250, 267). Interestingly, PDZ-RhoGEF co-localizes with Gα13 in these areas, suggesting that PDZ-RhoGEF may be involved in regulation of neuronal morphogenesis via the Gα13-coupled receptor. Jackson and colleagues showed that PDZ-RhoGEF specifically interacts with the intracellular C-terminal domain of excitatory amino acid transporter 4 (EAAT4) in Purkinje cells possibly to modulate the amount of glutamate in the synaptic cleft (267, 268). This finding suggests that the interaction between PDZ-RhoGEF and EAAT4 may be relevant to normal and abnormal glutamatergic neurotransmission (267), which may affect brain plasticity.
Type II diabetes is one of the most common chronic diseases in industrialized countries and is associated with high morbidity. Genetic and non-genetic factors have long been implicated in disease development (269, 270). In searching for genetic loci associated with a high prevalence of type II diabetes, a genome wide screen was carried out using various populations including Chinese, Caucasians, the Pima Indian of the Gila River Indian Community, and Old Order Amish (253, 270-273). These studies links type II diabetes, impaired glucose tolerance, and insulin resistance to chromosome 1q21-24, the region in which PDZ-RhoGEF (ARHGEF11) is located (253, 271, 273-275). More recently PDZ-RhoGEF (ARHGEF11) was also identified as a type II diabetes susceptibility gene in Old Order Amish and Pima Indian populations (276-278). Furthermore, a nonsynonymous single-nucleotide polymorphism in PDZ-RhoGEF (ARHGEF11) was found to be associated with a risk of lung cancer development (279). Both variants map to the C-terminus of PDZ-RhoGEF in the inhibitory region and this raises the possibility that changes in the guanine nucleotide exchange activity of PDZ-RhoGEF may relate to etiology of type II diabetes and cancer.

**Implication in human diseases with genetic alterations in Rho signaling**

The role of Rho-family GTPases in disease development has also been explored. Unlike Ras, mutations in Rho proteins are rarely found in tumors, instead their protein level or activity is often elevated in malignant cells. It has been demonstrated that constitutively active forms of RhoA and Rac1 exhibit oncogenic activity enabling fibroblast transformation but at a level that is
much less potent than Ras. However, activations of RhoA, Rac1, and Cdc42 are critically required for the oncogenic properties of Ras (280, 281). Deregulation of Rho-family GTPases protein level or activity has been implicated as a key factor in cancer initiation and progression (280-282). It was shown that RhoA, Cdc42 and Rac1 are critical components in neuronal development and perturbations in their activities or expression levels lead to cognitive disorders and neurodegeneration (283). Cdc42 and Rac regulate glucose transport and insulin secretion, indicating that these small GTPase also have roles in the development of metabolism-related disorders, such as diabetes and hypertension.

Thus far, the available evidence has demonstrated that Rho signaling plays a number of biological roles from regulation of mechanical support to signaling transduction, which has profound roles in maintaining physiological homeostasis. Given the numerous regulators and effectors that are involved in signaling inputs and biological outputs, investigation of the biological function governed by a specific Rho-family GTPase is a major challenge. Therefore, revealing the biological function of individual RGS-RhoGEF through RhoA GTPase is the initial step to unravel the signaling inputs, crosstalk between the Rho-family GTPases, and the effector-mediated biological and biochemical outputs.
**Thesis objectives**

A *Drosophila* RGS-Rho GEF, DRhoGEF2 was identified from a screen designed to identify genetic modifiers of the phenotype elicited by PTEN overexpression in *Drosophila* eye.

The first objective of this thesis was to characterize the biological function of DRhoGEF2 during eye development, to understand the effect of DRhoGEF2 on PTEN function, and to identify the mammalian ortholog of DRhoGEF2. The second objective was to explore the physiological role of PDZ-RhoGEF through generation of a PDZ-RhoGEF knockout mouse model.
Chapter II

DRhoGEF2, a Modulator of PTEN/PI3-Kinase signaling pathway during Drosophila melanogaster eye development
Abstract

The insulin/IGF-1 signaling pathway mediates various physiological processes associated with human health. The components of this pathway are highly conserved throughout eukaryotic evolution. In *Drosophila*, a PTEN ortholog (dPTEN) and its mammalian counterpart negatively regulate insulin/IGF signaling by antagonizing PI3-kinase function. In a genetic modifier screen for PTEN, we identified a gene that encodes DRhoGEF2, a guanine nucleotide exchange factor for Rho1 GTPase. DRhoGEF2 and its associated signaling cascade modify PTEN function genetically. *Drosophila* embryos with excess DRhoGEF2 display growth retardation and die at late second or early third instar larval stage. Overexpression of DRhoGEF2 disrupts compound eye development, which resulted in a cell type dependent eye phenotype. From a lethality rescue, we confirmed that a member of RGS-RhoGEFs, PDZ-RhoGEF (glutamate transporter EAAT4 associated protein, GTRAP48) is the closest mammalian ortholog that shares structural and function similarity with DRhoGEF2. Under the same control of expression, PDZ-RhoGEF recapitulated the compound eye phenotype seen in *Drosophila* with overexpression of DRhoGEF2. Furthermore, dAkt/dPKB phosphorylation in eye discs from wandering third instar larvae was increased when DRhoGEF2 mediated signaling was haploinsufficient, whereas, overexpression of DRhoGEF2 or PDZ-RhoGEF in proliferating neuronal precursor cells resulted in decreased dPKB/dAkt phosphorylation. We have demonstrated that conserved Rho signaling affects dPTEN function through dAkt/dPKB during *Drosophila* eye development. Moreover, our
identification of a structurally and functionally conserved mammalian ortholog raises the possibility that the effect of Rho signaling on PKB/Akt activation is also conserved in mammals.
**Introduction**

PTEN (phosphatase and tensin homology on chromosome 10 and also known as MMAC/TEP1) was identified on chromosome 10q23 and it is frequently deleted in advanced cancers. Through intensive studies during the past decade it has been found that PTEN, after p53, is the most frequently mutated human tumor suppressor gene. Both somatic mutations and germline mutations of PTEN have been identified (284). Loss of germline PTEN is directly linked to PTEN hamartoma tumor syndrome (PHTS). Somatic mutations, including gene deletion and gene inactivation of human PTEN, are found in a wide variety of human cancers such as brain, bladder, breast, prostate, endometrial, and skin cancers (285-291). Moreover, the high frequency of loss of heterozygosity for PTEN surpasses the frequency of biallelic inactivation, suggesting that haploinsufficiency for PTEN is adequate in promoting tumorigenesis in certain cellular contexts (292-299). It is well established that PTEN mechanistically functions as a PIP3 (phophatidylinositol-3,4,5-triphosphate) 3’-phosphatase to antagonize phosphoinoside 3-kinase (PI3-kinase), which elevates the level of intracellular PIP3. PIP3 recruits phosphoinoside-dependent protein kinase 1 (PDK1) and protein kinase B/mouse leukemia virus Akt8 (PKB/Akt) to the cytoplasmic membrane where PKB/Akt is activated by PDK1 and PDK2, mammalian target for rapamycin complex 2 (mTORC2) (300-305). PTEN as a tumor suppressor antagonizes PI3-kinase and represses cell proliferation through induction of apoptosis or cell cycle arrest (52, 286, 306-310), which results from the
inactivation of PKB/Akt, one of the downstream effectors of PI3-kinase. In addition, PTEN also modulates cell size, aging, polarity, and migration and its effects on these cellular processes have been conserved evolutionarily (311-319). In addition to the genetic mutations that have been identified, various non-genetically mediated mechanisms of suppressing PTEN function, including epigenetic silencing, post-translational modification, and changes in subcellular localization through protein-protein interaction, have been observed in various types of cancers (320-327).

It has been shown previously that an optimal level of dPTEN is vital during Drosophila and mouse embryogenesis (315, 328-330). Conserved dPTEN function has been characterized in a tissue-specific or cell-type specific fashion in both Drosophila compound eye and various tissues in mice (290, 312, 315, 331, 332). In Drosophila, the compound eye is not a vital organ for the organism. During eye development, cells in the eye imaginal disc undergo several distinct phases, such as cell proliferation, cell fate determination, cell death, cell growth, and differentiation. These processes are critical in generating functional photoreceptor cells and to maintain the precise architecture of the compound eye (Wolf and Ready, 1993). Importantly, PTEN has no effect on cell fate, instead, it controls timing of differentiation in Drosophila and axon guidance in C. elegans and mice, in addition to its role in regulating cell and organ size during eye development (332-335). Theses findings make Drosophila a powerful genetic model to investigate the regulation of PTEN which is conserved in Drosophila and mammals.
From a genetic screen in searching for genes that modify dPTEN function, a locus disrupted by a single P-element insertion encodes a member of Dbl family, DRhoGEF2 that partially rescued the small eye phenotype elicited by dPTEN-overexpression. DRhoGEF2, first identified as a member of the Dbl family, has been demonstrated to activate the Drosophila GDP/GTP exchange of DRho 1 (Drosophila ortholog of Rho-subfamily small GTPases) in two independent studies searching for components of Rho signaling and maternal effect during embryogenesis (234, 235). DRhoGEF2 regulates cell shape changes in the retina and gastrulation during embryogenesis via DRho1 GTPase and downstream of Folded gastrulation (Fog)/Concertina (Cta) (234, 235, 336-338). In this study, we have shown that DRhoGEF2/DRho1 signaling affects dPTEN function through dPKB/dAkt during eye development in a cell context dependent way. In addition, PDZ-RhoGEF, the mammalian ortholog of DRhoGEF2, shares not only protein sequence and structure homology but also displays functional similarity. Importantly, our data suggests that proper control of DRhoGEF2 signaling is necessary for maintaining ommatidia integrity of Drosophila compound eye and similar biological and biochemical effects of Rho signaling on PKB/Akt activation are conserved in both Drosophila and mammals.
Materials and Methods

Fly stocks

GMR-GAL4-UAS-dPTEN/CyO transgenic fly line was generated in our lab as described previously (339). The P-element line for DRhoGEF2 (cn1PRhoGEF204291/CyO; ry506 or DRhoGEF204291/CyO, stock number 11369), the driver lines, GMR-GAL4/II, EYE-GAL4/II, and Rho1 mutant line, w^aN^a-g, Drho1^E3,10/CyO (340) and rock^2/FM7 (141, 341), and a P-element enhancer line of RhoGAPp190 (y1w^67c23 P{EPgy2}RhoGAPp190^EY08765, stock number 20177) (342) were obtained from Bloomington Drosophila Stock Center at Indiana University.

DRhoGEF2^3w18/CyO, an EMS (Ethylmethanesulfonate)-induced mutant line, was kindly provided by Dr. Armen Manukian, Department of Medical Biophysics, University of Toronto (originally generated from Dr. Norbert Perrimon’s Laboratory at Harvard University) (235).
w^1118 was used as a wild-type control.

Transgene constructs and germline transformation

The 8.6 kb full-length DNA of DRhoGEF2 was cloned from a Drosophila melanogaster BAC clone containing DRhoGEF2 cDNA obtained from Research Genetics and subcloned into pUAST (339) and used to generate the pUAST-DRhoGEF2 transgenenic line by injecting into w^1118 embryos for germline transformation as described previously (343). Three DRhoGEF2 transgenic lines were generated (w;UAS-DRhoGEF2/CyO, w;UAS-DRhoGEF2/TM3, and w;UAS-DRhoGEF2/FM7).
**Rescue experiment**

A binary system was used to express transgene: $w^+; UAS$-$DRhoGEF2/CyO$ or $w^+; UAS$-$mycPDZ-RhoGEF/CyO$ in the fly in the presence of a $P$-element insertion mutant allele of $DRhoGEF2$, $w^+; DRhoGEF204291/CyO$ and chemically induced point mutation $w^+; DRhoGEF23w18/CyO$. Virgin females carrying $w^+; DRhoGEF2^{3w18}/CyO; ARM-GAL4/TM3$ were crossed to males carrying $w^+; DRhoGEF2^{3w18}/CyO; UAS$-$mycPDZ-RhoGEF/TM3$, or $w^+; DRhoGEF2^{3w18}/CyO; UAS$-$DRhoGEF2/TM3$. Only the genotype of F1 flies was assayed for viable adult flies. At least 2,000 flies were scored.

**Genetic crosses**

Standard genetic crosses were set up for ectopic expression of $DRhoGEF2$ in the fly eyes. $DRhoGEF2$ was overexpressed in the specific stage of eye development using the upstream activation sequence $(UAS)$-$GAL4$ binary system (339). During eye development, $GMR$-$GAL4$ (glass multiple reporter driven $GAL4$ expression) was employed to drive expression in the R cells in the eye imaginal disc and $Ey$-$GAL4$ (eyeless promoter driven $GAL4$ expression) was used to overexpress the transgenes in the anterior, undifferentiated region of the eye imaginal disc during the third instar larval stage (344).

**Ommatidial structure**

*Drosophila* eyes were fixed in 2% osmium/1% glutaraldehyde/0.1 M phosphate buffer (pH 7.2) for 30 min and followed by one change with fresh 2% osmium. After washing with 0.1
M phosphate buffer, osmium fixed eyes were dehydrated with ethanol and ethanol was replaced by propylene oxide. Eyes were embedded in Durcapan resin mixture (epoxy resin, hardner, accelerator, and plasticizer) in the modules for sectioning. Sections were stained with 1% toluidine blue solution.

**Immunohistochemical analysis for apoptosis and cell fate determination**

The eye imaginal discs were dissected from the third-instar larvae in S2 insect medium. Apoptosis was determined by staining with 3 µg/ml of acridine orange (Sigma-Aldrich). For cell proliferation, dissected discs were labeled with BrdU (bromodeoxyruidine, Becton Dickson) as described (345). Briefly, BrdU labeled eye discs fixed in PBS/4% para-formaldehyde (PFA), were denatured by HCl, and neutralized by PBS. Apoptosis were analyzed with a Zeiss fluorescent microscope. In order to generate gain-of-function clones, the FLP-out GAL4 system (flipase driven GAL4 expression) was employed (346). In brief, virgin females \( hsflp;act>y^+\rightarrow GAL4UASGFP/CyO \) were crossed with \( w^+;UAS-DRhoGEF2/UAS-DRhoGEF2 \) or \( w^+;UAS-mycPDZ-RhoGEF/UAS-mycPDZ-RhoGEF \) at 18°C for 3 days, then, parental flies were flipped out. Embryos were heat shocked for 45 min at 37°C and maintained at 25°C. Eye imaginal discs from wandering third-instar larvae were dissected and fixed in PBS/4% PFA (Sigma-Aldirch), washed in PBS/0.1% Triton X-100 (Sigma-Aldrich), and incubated overnight with primary antibody. Discs were stained with the following: rat anti-Elav (Developmental Studies Hybridoma Bank, University of Iowa)/goat-anti-rat-Cy5 (Jackson Lab), and phalloidin-
rhodamine (Molecular Probe). The stained discs were analyzed with a Zeiss confocal microscope

**Phenotypic and mosaic analysis of adult eyes**

All adult eye phenotypes were analyzed in females raised at 25°C unless indicated otherwise. The external eye phenotype was analyzed using a standard protocol for scanning electronic microscopy. For ommatidial organization, transverse sections were prepared for light and transmission electron microscopy.

**Protein analysis**

To prepare total protein lysates, five of eye imaginal discs were homogenized in cell lysis buffer (20 mM Tris (pH7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton x-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1mM phenylmethanesulfonyl fluoride (PMSF). Phosphorylation of dPKB/dAkt (serine 505), total dPKB/dAkt, and β-tubulin were detected using antibodies for phospho-S505 of dPKB/dAkt and total PKB/Akt (Cell Signaling Technology), β-tubulin (Upstate).
Results

**DRhoGEF2^{04291} as a suppressor of dPTEN overexpression induced developmental defects via dPKB/dAkt in Drosophila compound eyes**

A dominant modifier screen for mutations that affect the small eye phenotype resulting from *dPTEN* overexpression was conducted by crossing *dPTEN*-overexpressing flies (*w^{+};GMR-GAL4/UAS-dPTEN/CyO*) with a primary collection of 1045 *P*-element strains. Each strain comprises a single *P*-element insertion at one allele of each gene (347). Changes in the eye size of F1 progenies were scored for suppressing or enhancers of the small eye phenotype caused by overexpression of *dPTEN*. Of the 1045 *P*-element strains examined, we identified 6 strong suppressors and 14 strong enhancers that modified the eye size elicited by PTEN overexpression. One of the *P*-element insertions, *l(2)04291*, which maps to 53F01-2 locus on chromosome 2R (right arm of chromosome 2), partially rescued the small eye phenotype that resulted from overexpression of *dPTEN* (Figure II-1AII, Figure II-1AIII). From the *Drosophila melanogaster* genomic database, we identified that the *P*-element insertion, *l(2)04291*, inserts at the 5′-end of promoter region of *DRhoGEF2* locus and disrupted the expression of the corresponding allele of *DRhoGEF2* (referred to as *DRhoGEF2^{04291}* (234). To confirm the phenotype, fly strains with EMS disrupted alleles of *DRhoGEF2*, *DRhoGEF2^{3w18}* or *DRhoGEF2^{4.1}* (234, 235), which are complementary to *DRhoGEF2^{04291}* also suppressed the small eye phenotype mediated by *dPTEN* overexpression (Figure II-1AIV). It has been shown that overexpression of *dPTEN* in eye
Figure II-1. A P-element insertion mutation of DRhoGEF2 (DRhoGEF2^04291) acts as a dominant genetic modifier of dPTEN.

A(I-IV). Removing one copy of DRhoGEF2 by P-element disruption (DRhoGEF2^04291) or EMS induced point mutation (DRhoGEF2^5w18) suppressed the GMR-GAL4 driven UAS-dPTEN overexpression induced small eye phenotype. B. The ommatidial number of individual fly was determined by scanning electronic micrographs. In the presence of one copy of mutant allele of DRhoGEF2 had no effect on the number of ommatidia. (n = 10).

Genotype: (A1) w+;GMR-GAL4/+ (AII) w+;GMR-GAL4,UAS-dPTEN/CyO (AIII) w+;GMR-GAL4,UAS-dPTEN/DRhoGEF2^04291 (AIV) w+;GMR-GAL4,UAS-dPTEN/DRhoGEF2^5w18
imaginal discs increases apoptosis in differentiated cells and arrests proliferating cells at G2/M phase (315), resulting in the reduction of overall adult eye size. No difference in the number of ommatidia was found in *DRhoGEF*\(^{204291}\) flies, suggesting that loss of one allele of *DRhoGEF2* did not affect neuronal precursor cell proliferation during eye development (Figure II-1B). However, it became apparent that the flattened appearance with narrowed retina resulting from overexpression of *dPTEN* in differentiating cells was suppressed in the presence of one mutant allele of *DRhoGEF2* (Figure II-1Ab & Figure II-1Av).

It has been shown that deletion of *PTEN* leads to accumulation of PIP3 which in turn activates several signaling molecules including PDK1, PKB/Akt, S6 kinase, and mTORs, as well as small GTPase Cdc42 and Rac1 in mammals (52, 301, 304, 316, 348, 349). As one of the major effectors downstream of PI3-kinase, PKB/Akt activation is directly linked to elevated PIP3 in cell survival, proliferation, cell/organ size in both *Drosophila* and mammals (350-354). The phosphorylation status of dPKB/dAkt was examined in eye-imaginal discs prepared from third instar larvae of the *DRhoGEF2* mutant line. In *DRhoGEF2* mutant eye imaginal discs, we found that dPKB/dAkt phosphorylation was significantly increased at serine 505 (S505), a residue homologous to mammalian serine 473 of PKB/Akt that is required for full activation of PKB/Akt (Figure II-2A). This suggests that loss of one allele of *DRhoGEF2* resulted in increased dPKB/dAkt activation in eye imaginal discs during eye development. Considering that DRhoGEF2 activates the DRho1 mediated signaling cascade and DRok is one of the
Figure II-2. Reduction of DRhoGEF2 signaling increases activation of dPKB/dAkt and suppresses small eye phenotype elicited from dPTEN overexpression.

A. dPKB/dAkt phosphorylation was higher in the 3rd instar larval eye discs from P-element insertion mutant line (w\(^+\); DRhoGEF2\(^{04291}\)/CyO) than the wild type control w\(^{1118}\) (w\(^-\);+/+). B. Increased dPKB/dAkt activity was also observed in the 3rd instar larval eye discs in the presence of mutant allele of DRho1 (w\(^+\); Drho\(^{E3.10}\)/CyO) or Drok (Drok\(^2\)/FM7). C(I-II). The small eye phenotype induced by dPTEN overexpression was rescued when one mutant allele of DRho1 was introduced. D(I-II). Enhancer-promoter (EP)-induced overexpression of RhoGAPp190 suppressed dPTEN overexpression mediated small eye phenotype.

Genotype: (C1). w\(^+\)/y; GMR-GAL4, UAS-dPTEN/CyO (CII). w\(^+\)/y; GMR-GAL4, UAS-dPTEN/Drho\(^{E3.10}\) (DII). w\(^+\); GMR-GAL4, UAS-dPTEN/CyO (DII). w\(^+\); GMR-GAL4, UAS-dPTEN/CyO (DII). w\(^{67c23}\) P{EPgy2}\(\times\) RhoGAPp190\(\times\) Y08765; GMR-GAL4, UAS-dPTEN/+
common effectors downstream of DRho1, we further examined dPKB/dAkt phosphorylation at S505 in third instar eye imaginal discs isolated from \textit{Drho1}^{E3.10} and \textit{Drok}^2, mutant lines carrying a null mutant allele of each gene respectively. Phosphorylation of dPKB/dAkt at S505 was also increased in these imaginal eye discs (Figure II-2B). Thus, reducing DRhoGEF2 signaling increases phosphorylation of dPKB/dAkt at S505 during \textit{Drosophila} eye development. To further determine whether DRhoGEF2 signaling genetically interacts with dPTEN; we performed a genetic cross to express \textit{dPTEN} under \textit{GMR-GAL4} control in the presence of \textit{Drho1}^{E3.10}. Introducing one allele of \textit{Drho1}^{E3.10} resulted in a suppression effect and that was similar to the presence of a mutant allele of DRhoGEF2, \textit{DRhoGEF2}^{04191} or \textit{DRhoGEF2}^{3w18}, respectively (Figure II-2C). A similar suppression phenotype was also observed when DRho1 activity was reduced by introducing a mutant allele, enhancer-promoter (\textit{EP})-induced overexpression of \textit{RhoGAPp190} (\textit{RhoGAPp190}^{\text{EV08765}}) (Figure II-2D), suggesting that DRho1 activity has a major role in suppressing a small eye phenotype elicited by dPTEN overexpression. Importantly, these data demonstrate that DRho1 activity is counterbalanced by the upstream regulators, DRhoGEF2 and RhoGAPp190. Significantly, we have shown that first time that DRhoGEF2 signaling affects dPKB/dAkt activation.

**Identification and confirmation of the closest mammalian ortholog of DRhoGEF2**

It also has been shown that DRhoGEF2 shares domain sequence similarity with RGS-RhoGEFs, a subfamily of Dbl RhoGEFs, including PDZ-RhoGEF, LARG, and p115 RhoGEF.
The overall protein sequence similarity between DRhoGEF2 and RGS-RhoGEFs is about 20%, and, they not only share a similar functional domain structure with higher sequence similarity but also exhibit comparable substrate specificity. This suggests that mammalian members of RGS-RhoGEFs may have evolved from DRhoGEF2 and their function may be conserved throughout evolution. We further characterized the genetic relationship between DRhoGEF2 and RGS-RhoGEFs using unrooted phylogenetic analysis. By cluster alignment of the amino acid sequence of DRhoGEF2 and RGS-RhoGEFs, PDZ-RhoGEF was identified as potentially the closest mammalian counterpart (Figure II-3A). To further confirm, a genetic lethality rescue was performed using transgenic flies that carry transgene PDZ-RhoGEF or DRhoGEF2 under GAL4 (a transcriptional activator from yeast)/UAS (GAL4-binding site, upstream activation sequence) control in a tissue- or cell-specific pattern (339). ARM-GAL4-driven PDZ-RhoGEF or DRhoGEF2 expression was able to rescue the lethality caused by the presence of homozygous DRhoGEF2^{04291} (DRhoGEF2^{04291}/DRhoGEF2^{04291}) or heterozygous DRhoGEF2^{04291} with one allele of EMS allele DRhoGEF2^{3w18} (DRhoGEF2^{04291}/RhooGEF2^{3w18}). However, the alternative spliced isoform of PDZ-RhoGEF (PDZ-RhoGEFΔ8) was not able to rescue the lethal phenotype caused by a homozygous mutant allele of DRhoGEF2 (Figure II-3B). Accordingly, we were able to demonstrate that PDZ-RhoGEF is the mammalian ortholog of DRhoGEF2 by genetic approaches. Importantly, this data demonstrates that PDZ-RhoGEF can restore DRhoGEF2 mediated signal transduction during
Figure II-3. PDZ-RhoGEF is the mammalian ortholog of DRhoGEF2.

A. An unrooted phylogenetic analysis based on the ClustlW alignment of the amino acid sequence of five members of RGS-RhoGEF subfamily. The phylogenetic tree demonstrated that PDZ-RhoGEF is the closest mammalian ortholog of DRhoGEF2. B. PDZ-RhoGEF was further confirmed as the mammalian ortholog of DRhoGEF2 by genetic lethal rescue. Lethal phenotype resulted from homozygous mutation of \( DRhoGEF^{204291} \) was rescued by \( ARM-GAL4 \)-driven \( DRhoGEF2 \) or \( PDZ-RhoGEF \) expression. C. Embryos with \( ARM-GAL4 \) driven \( DRhoGEF2 \) or \( PDZ-RhoGEF \) overexpression exhibited growth retardation and died during late 2\(^{nd}\) or early 3\(^{rd}\) instar larval stage.
embryogenesis. In addition, embryos with either transgene PDZ-RhoGEF or DRhoGEF2 expression driven by GAL4 under control of ARM, a promoter for armadillo expression during early embryogenesis, displayed growth retardation and died at late 2\textsuperscript{nd} or early 3\textsuperscript{rd} instar larval stage (Figure II-3C). This may explain why the number of viable flies was much less than the Mendelian ratio.

**Role of DRhoGEF2/PDZ-RhoGEF in regulating cell proliferation and cell survival during early eye development**

Each compound eye comprises an almost perfect array of roughly 750 ~ 800 units of simple eyes known as ommatidia (Wolf and Ready, 1993). Each ommatidium is arranged in a stereotypical pattern, which includes a central core of eight photoreceptor cells referred to as retinula or R cells (R1-6 are known as the “outer” R cells while R7 and R8 are referred to as the ‘central’ R cells) surrounded by four cone cells (vertebrate lens in function), pigment cells and a sensory bristle. The adult eye develops from an epithelial monolayer known as the imaginal eye disc that invaginates from the ectoderm during embryogenesis and grows inside the larva (355). During the third instar of larval development, cells in the eye imaginal disc begin to pattern and differentiate progressively posterior to the morphogenetic furrow (MF), a transverse indentation. As furrow moves anteriorly into undifferentiated epithelium, cells posterior of the furrow are differentiated photoreceptors (R cells) (356-358). During pupation, while pigment cells are determined, all the cells undergo a series of important morphological and molecular changes that
lead to functional photoreceptors (terminal differentiated photoreceptors) (356, 358-360). During the last stage of eye development, apical membranes of photoreceptors elongate to form light-sensitive organelles (rhabdomeres) that allows photoreceptors to extend throughout the depth of the retina, then, photoreceptor axons establish synaptic connections into the optic lobe and rhodopsin as well as the transduction machinery are expressed and localized in the rhabdomeres and the ommatidium is completely formed (361, 362). Therefore, disruption of any these processes impairs the formation of functional photoreceptors and eventually lead to photoreceptor degeneration and an altered adult compound eye.

To determine the effect of D\(\text{RhoGEF2}\) overexpression on cell proliferation during eye development, we used a binary tissue-specific system, a tissue- or cell-specific phenotype generated by crossing two transgenic lines. Therefore, D\(\text{RhoGEF2}\) and PDZ-RhoGEF were ectopically expressed in cells or tissues where or when GAL4 was expressed. Expression of eyeless is restricted to the cells that divide in an asynchronous manner anterior to the morphogenetic furrow (MF) before differentiation during early eye development (363). Crossing UAS-D\(\text{RhoGEF2}\) or UAS-mycPDZ-RhoGEF to a transgenic line with GAL4 expression driven by the eyeless enhancer (\(E_{\text{y}}\)-GAL4), resulted in a reduced eye size (Figure II-4AII & 4AIII). A similar phenotype was observed in two independent transgenic lines that carry individual transgenes on either chromosome 2 or chromosome 3. We further examined the eye imaginal discs from early third instar larvae where UAS-D\(\text{RhoGEF2}\) expression was under the control of
Figure II-4. *Ey-GAL4* driven *DRhoGEF2* or *PDZ-RhoGEF* expression in undifferentiated epithelial cells in the eye disc resulted in small eye phenotype, increased apoptosis, and reduced dPKB/dAkt activity and protein expression.

**A(I-III)**. Scanning electronic micrographs demonstrated that overall adult eye size was reduced, when the ectopic expression of *DRhoGEF2* (A/I) or *PDZ-RhoGEF* (A/III) was under control of *Ey-GAL4*. **B(I-III)**. Increased apoptosis was detected by acridine orange (AO) staining in the eye disc with *Ey-GAL4* driven *DRhoGEF2* (B/I) or *PDZ-RhoGEF* (B/III) expression from 3rd instar larvae. **C(I-II)**. Overexpression of *DRhoGEF2* in undifferentiated epithelial cells resulted disorganized neuronal cell clusters (C/II). **D & E**. dPKB/dAkt protein level and phosphorylation at S505 were reduced in the 3rd instar larval eye disc with *Ey-GAL4* driven *DRhoGEF2* (D) or *Ey-GAL4* driven *PDZ-RhoGEF* expression (E). A: antennal disc, E: eye disc.

Genotype: (I). w<sup>+</sup>; +/-; *Ey-GAL4*+/ (II). w<sup>+</sup>; UAS-*DRhoGEF2*+; *Ey-GAL4*/+ (III). w<sup>+</sup>; UAS-*mycPDZ-RhoGEF*+/; *Ey-GAL4*/+
Ey-GAL4. Changing of eye size might imply that Ey-GAL4 driven ectopic expression of DRhoGEF2 negatively regulates cell growth, proliferation or survival in the cells anterior to the MF, which might contribute to the cell fate determination during early eye development. For cell survival, there was a significant increase in acridine orange (AO) positive cells detected in both DRhoGEF2- and PDZ-RhoGEF-overexpressing cells anterior to the MF (Figure II-4BII & II-4BIII). The disorganized neuronal cell clusters were demonstrated by staining with an antibody for a neuron specific transcription factor, Elav (Figure II-4CII & II-4CIII). Significantly, dPKB/dAkt phosphorylation at S505 and its protein levels were decreased when DRhoGEF2 or PDZ-RhoGEF were expressed specifically in proliferating cells during early eye development (Figure II-4D & II-4E). The decreased dPKB/dAkt phosphorylation and protein level may account for the increased apoptosis in neuronal precursor cells, which was also observed in the presence of mutant dPKB/dAkt during embryogenesis (350, 352).

The effect of elevated DRhoGEF2/PDZ-RhoGEF levels on the regulation of photoreceptor differentiation

GMR (glass multimer reporter) is a promoter region for the transcription factor glass, which is initially expressed in the post-mitotic cells that are located posterior to the MF and is maintained during late larval and early pupal stages of eye development. The cells posterior to the MF are progressively differentiated into photoreceptors (R1 to R8) and other accessory cells. Therefore, DRhoGEF2/PDZ-RhoGEF overexpression driven by GMR-GAL4 was restricted to
differentiated cells. The adult eye phenotype of *Drosophila* resulted from *DRhoGEF2/PDZ-RhoGEF* overexpression driven by *GMRGAL4* in post-mitotic cells was different from the phenotype that resulted from *DRhoGEF2/PDZ-RhoGEF* overexpression in proliferating cells (Figure II-4A). Overexpression of *DRhoGEF2/PDZ-RhoGEF* driven by *GMR-GAL4* disrupted the outer ommatidial lattice with loss of bristles resulting in an extremely rough and smaller eye phenotype (Figure II-5A-II & II-5A-III). The phenotype was confirmed by two independent transgenic lines, suggesting that the insertion sites had no effect on the eye phenotype. To characterize the cellular abnormalities in the rough eyes, toluidine stained transverse sections of the adult compound eyes were analyzed by light microscopy (Figure II-5B-II & II-5B-III). In contrast to the control sections (Figure II-5B-I), overexpression of *DRhoGEF2/PDZ-RhoGEF* disrupted well-organized ommatidial lattice and sections displayed vesicles containing rhabdomere remnants, suggesting that the photoreceptor and accessory cell pattern formation was possibly affected during eye development. Interestingly, there was no difference in both proliferation and cell survival between *DRhoGEF2/PDZ-RhoGEF*-overexpressing and control eye discs by acridine orange (AO) staining (Figure II-5C-I, 5C-II, & 5C-III) and BrdU uptake (Figure II-5D-I, 5D-II, & 5D-III).

Next, we employed heat shock-induced mitotic recombination to clonally overexpress *DRhoGEF2/PDZ-RhoGEF* during early development in 3rd instar larvae. Recombination and gene expression was induced by actin driven *GAL4* expression after heat shock. Three days after
Figure II-5. GMR-GAL4-driven DRhoGEF2 or PDZ-RhoGEF expression in the differentiated cells resulted in rough eye phenotype.

A(I-III). Scanning electron micrographs demonstrated rough eye phenotype resulting from ectopic expression of DRhoGEF2 (AII) or PDZ-RhoGEF (AIII) under the control of GMR-GAL4. B(I-III). The rough eye phenotype was due to disrupted ommatidial integrity observed by toluidine blue-stained transverse sections of the adult eye, when DRhoGEF2 (BI) or PDZ-RhoGEF (BIII) was overexpressed in photoreceptor cells. C(I-III). Overexpression of DRhoGEF2 (CI) or PDZ-RhoGEF (CIII) displayed no difference in apoptosis in the 3rd instar larval eye discs by acridine orange (AO) staining. D(I-III). Overexpression of DRhoGEF2 or PDZ-RhoGEF in differentiated neuronal cells had no effect on cell proliferation in the 3rd instar larvae. Cell proliferation was determined by BrdU incorporation. A: antennal disc, E: eye disc.

Genotype: (i). w+; GMR-GAL4/+  (ii). w+; GMR-GAL4 UAS-DRhoGEF2  
(iii). w+; GMR-GAL4 UAS-mycPDZ-RhoGEF/+
heat shock, eye imaginal discs were prepared from wandering 3rd instar larvae, followed by immunoflorescent staining with Elav antibody to examine neuronal cell fate and then counterstained with phalloidin to examine actin organization. DRhoGEF2/PDZ-RhoGEF overexpression was marked by expression of green fluorescent protein (GFP) (Figure II-6A & II-6B), after heat-shock induced recombination occurred in the eye imaginal discs. We examined the cell fate determination using the neuronal marker, Elav. Compare to the neighboring control cluster of neuronal cells (GFP-negative area), the organization of neuron cell clusters was not interrupted (Figure II-6AII & II-6BII). In addition, the actin cytoskeleton organization was not affected by either of the transgenes (Figure II-6AIII & II-6BIII). Evidently, overexpression of DRhoGEF2 or PDZ-RhoGEF in post-mitotic neuronal cells had no effect on neuronal cell fate determination (Figure II-6AI & II-6BII). Not surprisingly, there was no difference in phosphorylation of dPKB/dAkt at S505 detected in eye discs from 3rd instar larvae (Figure II-6C & II-6D). These observations indicate that overexpression of DRhoGEF2/PDZ-RhoGEF in the post-mitotic neuronal cells neither affected cell-fate determination nor actin cytoskeleton organization in retinal cells at the wandering 3rd instar larval stage.

**Essential role of DRho1 downstream of DRhoGEF2/PDZ-RhoGEF in photoreceptor differentiation**

To further confirm that DRho1 plays an essential role in establishing the rough eye phenotype downstream of both DRhoGEF2 and PDZ-RhoGEF, a mutant line carrying
Figure II-6. Overexpression of DRhoGEF2 or PDZ-RhoGEF has no effect on cell fate determination and dPKB/dAkt phosphorylation and total dPKB/dAkt protein.

A&B. The cell fate determination was not impaired when DRhoGEF2 (A) or PDZ-RhoGEF (B) overexpressed by heat-shock induced mitotic recombination in the post-mitotic neuronal cells.

C&D. The levels of phospho-S505 of dPKB/dAkt and total dPKB/dAkt protein were not affected when DRhoGEF2 (C) or PDZ-RhoGEF (D) were overexpressed in the 3rd instar eye imaginal discs under GMR-GAL4 control.

Genotype: (A) hsflp:act,FRT,GAL4-UAS-GFP/UAS-DRhoGEF2 (B) hsflp:act,FRT,GAL4-UAS-GFP/UAS-mycPDZ-RhoGEF
Rho1 mutation ($Drho1^{E3.1}$) was used to reduce the gene dose of endogenous Drho1 by half. The external ommatidial structure of an adult fly eye resulting from GMR-GAL4 driven DRhoGEF2 expression could fully be rescued in the presence of $Drho1^{E3.1}$ (Figure II-7AII). In addition, a Dominant negative form of Cdc42 and Rac1 (Cdc42$^{N17}$ and Rac1$^{N17}$) failed to suppress the DRhoGEF2 induced rough eye phenotype (Figure II-7AIII & II-7AIV). This data demonstrates that DRho1 activity is critical for maintaining ommatidia integrity. Previously, we showed that RhoGAPp190 suppressed the dPTEN-dependent small eye phenotype, suggesting that RhoGAPp190 may antagonize DRhoGEF2 signaling by facilitating the intrinsic rate of GTP hydrolysis of DRho1. Indeed, DRhoGEF2 was partially rescued when RhoGAPp190 was co-expressed under GMR-GAL4 control (Figure 7A-III), suggesting that RhoGAPp190 can partially antagonize DRhoGEF2 activity which suppresses DRho1 activity. However, the rough eye phenotype (Figure II-7BII) resulting from overexpression of PDZ-RhoGEF by GMR-GAL4 was particularly suppressed when $Drho1^{3E.10}$ was introduced (Figure II-7BIII) or RhoGAPp190 was co-expressed (Figure 7BIII).
Figure II-7. Reduction of DRho1 activity rescued the rough eye phenotype resulting from GMR-GAL4 driven DRhoGEF2 and PDZ-RhoGEF overexpression.

A. Reduced DRho1 activity rescued rough eye phenotype resulting from GMR-GAL4-driven DRhoGEF2 overexpression in the presence of one allele of mutant DRho1, Drho13E.10 (Aii). Overexpression of dominant active Rac1 (Aiii) or Cdc42 (Aiv) could not rescue DRhoGEF2 overexpression induced rough eye phenotype. The partially rescued eye phenotype was observed when RhoGAPp190 was co-expressed with DRhoGEF2 under GMR-GAL4 control (Av). B. The PDZ-RhoGEF overexpression that induced rough eye phenotype was partially rescued when Drho13E.10 was introduced (Bii) or when the expression level of RhoGAPp190 was elevated (Biii).

**Discussion**

DRhoGEF2 has been suggested to be a member of the Dbl family functioning to transmit Gα-protein coupled receptor (Fog/Cta) dependent and independent signals through DRho1 to regulate cell shape, invagination, and epithelial folding during *Drosophila* embryogenesis and eye development (234, 235, 336-338). In this study, we have specifically demonstrated that DRhoGEF2, a regulator of *Drosophila* Rho signaling, genetically interacts with *dPTEN*, through its downstream target, DRho1. Moreover, we demonstrated that the outcome of overexpression of DRhoGEF2 is cell-context dependent, contributing to a distinct eye phenotype. These data suggest that ectopic expression of DRhoGEF2 may have temporal effects on *Drosophila* eye development. The balance between the active and inactive form of DRho1 modulated by DRhoGEF2 and RhoGAPp190 is important in maintaining ommatidial lattice integrity. Importantly, we have demonstrated for the first time that the DRhoGEF2 and PDZ-RhoGEF share functional similarity in maintaining ommatidia integrity via enhancing DRho1 activation. Together, these studies have demonstrated that the interaction between components in two conserved signaling pathways, DRhoGEF2/DRho1 and PI3-kinase/dPKB/dAkt, plays a crucial role in neuronal cell differentiation during *Drosophila* eye development.

**Genetic interaction between DRhoGEF2-mediated signaling and dPTEN**

We have shown that dPTEN overexpression induces a small eye phenotype and that this can be suppressed in the presence of DRhoGEF2 mutants or by mutant forms of downstream
effectors, including null mutation of both DRho1 and DRok. These data suggest that the reduction of DRhoGEF2 signaling partially suppresses PTEN function. It has been shown that both inactivation and overexpression of Drosophila PTEN affects cell size, while overexpression of dPTEN also inhibits cell cycle progression at early mitosis and promotes cell death during eye development in a context-dependent manner (315), resulting in decreased eye size phenotype. Therefore, loss of one allele of DRhoGEF2 had no effect on total number of ommatidia from an eye with dPTEN overexpression, suggesting that DRhoGEF2 may have no effect on apoptosis induced by GMR-GAL4 driven dPTEN overexpression. We have shown that potentially reduced DRhoGEF2 signaling activates dPKB/dAkt and rescued the flattened and narrowed retina, resulting from change in cell shape and size induced by GMR-GAL4 driven dPTEN expression in differentiating cells. Barret and colleagues have shown that the mutant form of DRhoGEF2 suppressed GMR-Rho1 induced rough eye phenotype specifically by restoring retinal cell elongation (234). An increased retinal cell elongation was also observed in the retina with GMR-GAL4 driven wild type Drosophila expressing PI3-kinase Dp110, whereas, overexpression of dominant-negative Dp110 (Dp110D954A) reduced the thickness of the retina (364). Therefore, the effects of reduced DRhoGEF2 signaling mediated dPKB/dAkt activation may recap the retinal cell elongation resulting from overexpression of wild type Dp110. Here, we have shown a member of small GTPase of Rho family, DRho1 and its regulator, DRhoGEF2 may interact with PI3-kinase signaling to mediate retinal structure.
Cell-context-dependent effects of DRhoGEF2 in the Drosophila visual system

Although DRhoGEF2 overexpression results in a very distinct phenotype, cell fate determination was not affected under either eyeless enhancer or glass promoter control. The distorted neuronal cell cluster was a secondary effect resulting from an extensive apoptosis when the expression levels of DRhoGEF2 were elevated by Ey-GAL4 in proliferating cells located anterior to the MF. These data suggest that the phenotypic divergence of adult eye depends on the timing of DRhoGEF2 upregulation during eye development. Therefore, DRhoGEF2 signaling needs to be tightly controlled temporally in order to maintain the precise cellular architecture of the Drosophila retina. We have shown that Ey-GAL4 driven UAS-DRhoGEF2 overexpression resulted in a decrease in dPKB/dAkt activity and the reduction of dPKB/dAkt protein level. It has been firmly established that loss-of-function mutations of components of the insulin/IGF-1 pathway, including the insulin receptor (InR), Chico, PI3-kinase, and dPKB/dAkt, lead to defects in cell growth during Drosophila eye and wing development (312, 342, 354, 365) as well as cell survival during Drosophila embryogenesis (351). Moreover, dPTEN mutant clones generated in the early 1st–instar stage, a proliferating stage, resulted in three times as many as mutant cells than wild type twin spots (331). Thus, reduced protein levels and phosphorylation of dPKB/dAkt resulting from elevated DRhoGEF2 expression in neuronal precursor cells may alter cell survival and cell number, and therefore affect the total number of ommatidia and the overall size of the adult eye.
Surprisingly, overexpression of DRhoGEF2 in differentiated neuronal cells resulted a rough eye phenotype with impaired terminal photoreceptor differentiation (Figure II-5), which was similar to the phenotype resulted from GMR-driven DRho1 overexpression in differentiated neuronal cells (397). Importantly, overexpression of DRhoGEF2 had no effect on differentiated neuronal cell proliferation, cell survival, cell fate determination, as well as the phosphorylation and protein levels of dPKB/dAkt in the 3rd instar larval eye imaginal discs (Figure II-5 & Figure II-6). Together, these findings suggest that overexpression of DRhoGEF2 possibly has no effect on pattern formation at 3rd instar larval stage but it affects photoreceptor pattern formation and terminal differentiation during pupariation, a stage that eye development continues to specify new cell types, including cone cells, pigment cells, and organize them into the precise retinal cell lattice (357).

**Potential role of constitutively activated Rho signaling and resulting retinal degeneration**

The differentiation of photoreceptors is a continuous process from larval to pupal stage. During pupation, specified photoreceptors and cone cells undergo a series of morphological changes such as establishing synaptic connections, rhabdomere morphogenesis, and expression of rhodopsin and the transduction machinery, during pigment cell determination (366). Importantly, disruption of any of these processes blocks the formation of functional photoreceptors and eventually leads to photoreceptor degeneration (366). In GMR-GAL4 driven
UAS-DRhoGEF2/UAS-mycPDZ-RhoGEF overexpression eye discs from 3rd instar larvae, dPKB/dAkt phosphorylation and protein levels were not affected, which may relate to an undisturbed imaginal eye disc. Therefore, there was no obvious difference in apoptosis or proliferation levels compare to control (GMR-GAL4). However, the external and internal ommatidial structures were severely impaired, suggesting that the effect on eye development is at a process further than cell fate determination. The disruption of ommatidial architecture by DRhoGEF2 or PDZ-RhoGEF overexpression was similar to the retinal degeneration caused by rhodopsin 1 mutation, GMR-driven overexpression of Drho1 or a constitutively active form of DRok-cat (N-terminal kinase region) (367-369). Overexpression of a baculoviral caspase inhibitor p35 under GMR was able to rescue the rhodopsin 1 mutation, DRho1 as well as DRok-cat overexpression mediated retinal degeneration (368-373), suggesting that a caspase-dependent apoptotic pathway is involved in constitutively activated Rho signaling or dysfunctional G-protein coupled receptor (rhodopsin) induced programmed cell death in Drosophila retina. However, co-expression of GMR-p35 did not rescue the rough eye phenotype resulted by GMR-GAL4 driven UAS-DRhoGEF2 or UAS-mycPDZ-RhoGEF overexpression. It is possible that enhanced GDP/GTP exchange of DRho1 GTPase by DRhoGEF2/PDZ-RhoGEF or multiple effectors downstream of DRho1 contribute to the deterioration of ommatidia; however, a gene dosage effect cannot be excluded.

For the past decade, a conserved role of the Rho-family of small GTPases in various
aspects of neuronal development via regulation of actin cytoskeleton and microtubule stability has emerged (283). It has been shown that Rac1 and cdc42 affect axonal extension and branching in *Drosophila* motor neuron, the giant fiber system, and peripheral neurons (36, 374, 375). On the other hand, mice lacking *p190 RhoGAP* exhibit a specific reduction in subcortical axon number, which may be due to inhibition of axon initiation and/or axon retraction due to elevated RhoA activity (84). There is no direct evidence to implicate that *Drosophila* Rho1 signaling is involved in axon retraction in *Drosophila* photoreceptor axonal extension, however, it has been demonstrated indirectly that inactivation of RhoGAPp190 in *Drosophila* mushroom brain neurons causes axon branch retraction that was mimicked by constitutively activated DRok (376). This observation suggests that *Drosophila* Rho1 signaling may also have a negative role in axonal extension. We have shown that reduced gene dosage of *Drho1* can fully rescue the rough eye phenotype induced by *GMR-GAL4* driven *UAS-DRhoGEF2* overexpression. In contrast, the rough eye phenotype was partially rescued or suppressed by co-expression of *RhoGAPp190*, which may result from dosage-dependent effects or the intrinsic GTPase activity of DRho1, which is regulated by multiple Rho GAPs. Nonetheless, these observations raise the possibility that DRhoGEF2-mediated constitutive activation of DRho1 signaling may promote growth cone collapse to stall axonal extension. As a result, it is possible that a failure of axonal extension prevents photoreceptor maturation and blocks the synaptic connection between photoreceptor axons and the optic lobe, leading to neuron degeneration in a caspase-dependent
and independent manner.

**Possible role of DRhoGEF2-dependent signaling in axonal guidance**

We have shown that overexpression of PDZ-RhoGEF restored DRho1 activation-mediated embryogenesis in the absence of DRhoGEF2 and recapitulated the cell-context-dependent effects on *Drosophila* eye phenotype elicited by DRhoGEF2 overexpression. This finding has further alluded to the likelihood that PDZ-RhoGEF and DRhoGEF2 are functional analogues coupling Gα-coupled receptor signaling to RhoA activation in *Drosophila* and mammals (Figure II-8A). In mammals, Gα12/13-coupled receptor signaling is involved in RhoA-regulated neurite retraction for proper positioning of migrating cortical neurons (88, 377, 378). Although the role of PDZ-RhoGEF in Gα12/13-regulated neurite retraction needs to be further elucidated, PDZ-RhoGEF has been implicated in semaphorin 4D/plexin B1-mediated growth cone collapse or neurite retraction in rat hippocampal neurons through RhoA activation (Figure II-8B) (249). Given that various guidance cues are conserved in *Drosophila* and mammals (283), DRhoGEF2 may also couple these cues to DRho1-mediated axon guidance in a cell-context-dependent manner. We have shown the potential association between DRhoGEF2 signaling and dPKB/dAkt activity during photoreceptor development; a negative effect on dPKB/dAkt activity in neuronal precursor cells. Interestingly, small GTPase RhoA and its downstream effectors, ROCKs, have been suggested in negatively regulating PI3-kinase signaling via enhancing PTEN lipid phosphatase activity in neutrophil chemotaxis (325, 379), axon growth and sensory neuron
Figure II-8. Possible role of DRhoGEF2 and PDZ-RhoGEF as signaling integrators downstream of repulsive and guidance cues in negative regulating PI3-kinase-dPKB/dAkt mediated axon guidance during *Drosophila* eye development.

A. G$_{12}$-coupled receptor. B. semaphorin/plexin-mediated repulsive cue. C & D. Proposed role of insulin-like receptor in axon guidance. E. Possible role of PTEN as an effector downstream of DRok in negative regulating PI3-kinase/dPKB/dAkt activation-mediated axon guidance downstream of *Drosophila* insulin-like receptor. (Modified from ref 371)
regeneration (380). Interestingly, the role of PI3-kinase signaling in axon guidance downstream of various guidance molecules has been shown in *Xenopus* (381, 383). Particularly, guidance molecules, such as netrin-1, trigger PI3-kinase signaling-mediated protein synthesis, which plays an essential role in *Xenopus* retinal axon guidance (381-383). Therefore, it is possible that insulin-like receptor signaling may also regulate axon guidance through enhancing protein synthesis via activation of PI3-kinase signaling (384) (Figure II-8C). Furthermore, Song and colleagues has shown that insulin-like receptor signaling functions as a guidance cue in regulating axon guidance through Dock/Rac1-mediated Pak activation (381, 382) (Figure II-8D). It has been shown that p21-activated kinase (PAK), a Ser/Thr kinase downstream of Rac and Cdc42, is localized to the neutrophil in developing optic lobes (35). Considering that the opposite role has been identified between *Drosophila* Rho1 and Rac/Cdc42 in regulating axonal guidance and neurite outgrowth (283), it is possible that DRho1 and DRok are also expressed in the neutrophil to counterbalance DCdc42 and DRac1 (Figure II-8D). Collectively, we hypothesize that overexpression of DRhoGEF2/PDZ-RhoGEF may negatively regulate PI3-kinase-dPKB/dAkt signaling cascade leading to disruption of axon at a late stage of photoreceptor differentiation (Figure II-8C). Thus, characterizing the subcellular localization of DRho1, DRok, and dPKB/dAkt may shed light on how DRhoGEF2 signaling mediates dPKB/dAkt activity in the developing visual system. Particularly, unraveling the mechanistic effect of DRho1/DRok on dPKB/dAkt activity should allow us to determine the role of DRhoGEF2 in Go-coupled
receptor, semaphorin 4D/plexin-B, and/or insulin/DInR signal transduction modulated retinal axon guidance (Figure II-8E).

**The implication of PDZ-RhoGEF in brain plasticity – from Drosophila to human**

Several studies have suggested that DRhoGEF2 is required for the convergence of multiple pathways to complete ventral furrow formation during *Drosophila* embryogenesis (234, 235, 338, 385-387). The salvage of *Drosophila* embryonic lethality by human *PDZ-RhoGEF* suggests that human PDZ-RhoGEF, as part of a Gα-coupled-receptor signaling pathway, restores signal transduction and biological functions that are mediated by DRhoGEF2 to DRho1 activation during embryogenesis. Strikingly, overexpression of PDZ-RhoGEF can recapitulate the mechanistic and phenotypic effect of DRhoGEF2 overexpression during *Drosophila* eye development. Several studies have also shown that PDZ-RhoGEF expression is found in hippocampal neurons and Purkinje cells (250, 267), two areas which have been linked to various neurodegenerative diseases (388, 389). Importantly, the expression of PDZ-RhoGEF is differentially elevated in patients suffering from schizophrenia (390, 391), suggesting that altered PDZ-RhoGEF expression in certain areas of the brain may contribute to neuronal disorders.

Consistent with this finding, our *Drosophila* model has demonstrated that overexpression of DRhoGEF2/PDZ-RhoGEF disrupts neuronal development during *Drosophila* eye development. Therefore, it raises the possibility that PDZ-RhoGEF mediated signaling cascade could have gain-of-function effects on brain plasticity.
Chapter III

PDZ-RhoGEF regulates cell proliferation

and

adipose tissue mass
Abstract

In this study, we explored the physiological function of a RhoA-specific guanine nucleotide exchange factor (GEF) PDZ-RhoGEF (ARHGEF11), by disrupting the ARHGEF11 gene in mouse. We demonstrated that PDZ-RhoGEF is dispensable for a number of known RhoA signaling-mediated processes, including embryogenesis, stress fiber formation and cell migration. However, PDZ-RhoGEF deletion led to a reduction in adipose tissue mass and protected animals from diet-induced obesity and insulin resistance. PDZ-RhoGEF was essential for the proliferative response to insulin/IGF-1 in white fat and mouse embryonic fibroblasts (MEFs) and when overexpressed led to cell transformation. Mechanistically, PDZ-RhoGEF mediated ROCK-dependent phosphorylation of the insulin receptor substrate-1 (IRS-1) necessary for optimal insulin/IGF-1 signaling throughput. Taken together, our results implicate PDZ-RhoGEF as a major physiological modulator of insulin/IGF-1 signaling and a key factor in adipocyte homeostasis.
Introduction

Insulin/IGF-1 signaling has pleiotropic effects on growth, development, metabolic homeostasis, as well as aging via receptor tyrosine kinases, insulin receptor (IR) and IGF-1R activated signaling cascades (392-408). In brief, ligand-activated IR and IGF-1R phosphorylates IRSs at tyrosine residues and thereby recruits various SH2-containing signaling proteins, including p85 (the regulatory subunit of PI3-kinase), growth factor receptor bound protein 2 (Grb2), SH2-containing phosphatase-2, (SHP), isoforms of SH2-containing protein (Shc), and c-Cbl-associated protein (CAP), to transduce insulin or IGF-1 action. Via these distinct adaptor molecules, insulin/IGF-1 signaling triggers signaling cascades that are initiated by PI3-kinase, small GTPase Ras, and c-Cbl. Among all the adaptor proteins, IRS-1 and IRS-2 are the common elements in transmitting the signals from ligand-activated IR and IGF-1R to activate PI3-kinase/PKB/Akt signaling (409-412). PTEN, a lipid phosphatase, down-regulates the level of lipid second messenger PIP3 generated by insulin/IGF-activated PI3-kinase, which represses cell proliferation through induction of apoptosis or cell cycle arrest and affects cell size, polarity, migration, as well as aging (286, 306-314, 316-319, 413).

Mutations that alter insulin/IGF signaling have severely detrimental effects on human health conditions that shorten life span, such as insulin resistance, obesity, diabetes, atherosclerosis, and cancer (414-419). Insulin resistance, a pathological state in which cells or tissues fail to respond to normal circulating levels of insulin, has been tightly linked to obesity
Obesity is a disease state that results from a complex interaction between genetic predisposition towards an expansion of adipose tissue mass and consequences of adaptation to the environment, such as diet. Therefore, research in understanding the molecular mechanisms involved in controlling adipose tissue expansion and development have been undertaken. Initially, a dense mass of mesenchymal cells associated with vascular structures appears at areas where the multipotent mesenchymal or adipose tissue stem cells undergo cell fate determination and commitment to become lineage-determined preadipocytes. Eventually, the preadipocytes are converted into mature, lipid-filled, and insulin sensitive adipocytes as a result of adipogenic stimulation, including insulin and IGF-1. Adipose tissue growth results from an increase in both adipocyte cell size and adipocyte cell number, known as adipogenesis, and occurs as part of normal body growth, as well as during the development of obesity. Increased adipocyte size is the result of excess triglyceride accumulation in differentiated adipocytes due to the accumulation of additional triglyceride from dietary sources. Increases in the number of adipocytes is attributable to the differentiation of resident preadipocytes, precursors derived from the resident stromal-vascular compartment of mature adipose tissue, and/or mesenchymal progenitor cells recruited from non-residence sources, such as bone marrow. Interestingly, it has been demonstrated that RhoA/Rho-kinase signaling directs cells from mesenchymal origin to commit into different lineages, such as adipocytes, myoblasts, and osteoblasts. These findings suggest that the regulation of
RhoA GTPase activity is critical in adipogenic lineage determination, which in turn affects adipose tissue expansion.

From a genome-wide linkage scan, ARHGEF11 and ARHGEF12, which encode PDZ-RhoGEF and LARG, respectively, were identified as positional candidate genes for susceptibility to type II diabetes (254, 276, 277, 446). Genetic variants in PDZ-RhoGEF and LARG were identified, and have been associated with glucose tolerance, insulin sensitivity, and type II diabetes. PDZ-RhoGEF and LARG are RhoA specific guanine nucleotide exchange factors (222, 241). PDZ-RhoGEF shares similar domain architecture with LARG, including a PDZ domain, a RGS-like domain, a catalytic DH-PH domain, and an additional proline-rich domain at the C-terminus of the PDZ domain (222, 225). Accumulating evidence indicates that PDZ-RhoGEF and LARG transmit a partially overlapping set of signals to RhoA, suggesting functional redundancy between these two molecules (244, 249, 257, 258, 447, 448). This, coupled with their relatively broad tissue expression, complicates the assessment of each in terms of specific physiological roles.

To elucidate the specific role of PDZ-RhoGEF in vivo, in this study, we explored the systemic function of PDZ-RhoGEF by disrupting its gene in mouse. While our data demonstrate that PDZ-RhoGEF is dispensable for many signaling inputs previously linked to its regulation, we discovered its direct involvement in controlling adipose tissue homeostasis. Furthermore, we have shown that lack of PDZ-RhoGEF prevents diet-induced obesity and insulin resistance in
in vivo. Elevated PDZ-RhoGEF results in RhoA/ROCK dependent cellular transformation through sustained cell-cycle progression and inhibition of apoptosis. Mechanistically, PDZ-RhoGEF was found to induce RhoA/ROCK-dependant phosphorylation of IRS-1, which was required for optimal insulin/IGF-1 signaling throughput and oncogenic functions.
Materials and Methods

Expression constructs

Full length PDZ-RhoGEF (ARHGEF11 variant 2) was first amplified from a human brain cDNA library (Clontech). An alternatively spliced isoform of PDZ-RhoGEFΔ8 (ARHGEF variant 1, lack of exon 8) was engineered by polymerase chain reaction from full-length PDZ-RhoGEF using specific primers. Subsequently, both constructs were subcloned into a retroviral vector, pBMN-IRES-GFP (from Dr. Gary Nolan, Stanford University) to generate stable cell lines and pcDNA3.1-N-myc or pcDNA3.1-N-flag (Invitrogen) for transient transfection. GST (Glutathione-S-transferase) recombinant fusion protein GST- PDZ-RhoGEFΔC (aa 1-735) was generated and subcloned into a bacterial expression vector pGEX-4T.1 (Amersham Pharmacia Biotech) for making a polyclonal rabbit anti-PDZ-RhoGEF antibody (TC-1) (Department of Comparative Medicine, University of Toronto).

Generation of stable cell lines

The cells were cultured in complete medium (DMEM/10% fetal bovine serum /2 mM L-glutamine/1 mM sodium pyruvate/0.055 mM β-mercaptoethanol). NIH-3T3 cells were purchased from American Type Culture Collection (ATCC). Transient transfection and infection were done as described (449). Briefly, retroviral packaging Phoenix E cells were plated and transfected with retroviral constructs by calcium phosphate precipitation. Viral supernatant was collected at 48 and 72 hours post transfection. NIH-3T3 cells were spin infected with viral
supernatant at room temperature. Infected cells were expanded at 24 hours after infection. Infection efficiency was determined by percent of green fluorescent cells using FACS Calibur (BD Biosciences).

Cell proliferation and cell cycle re-entry analysis

For proliferation assay, cells were plated and starved with 0.5% FCS or 0% FCS for 24 hours or 48 hours. Proliferation was scored by measuring $[^3]$H-thymidine uptake in response to mitogens, IGF-1 (75 ng/ml), epidermal growth factor (EGF, 75 ng/ml), lysophosphatidic acid (LPA)/dimethyl sulfoxide (DMSO, 20 µM), and FCS (10%). S phase entry was determined by propidium iodide staining at each time point upon stimulation. Cell cycle progression data was collected by FACS Calibur (BD Biosciences, CA) and analyzed using Flow-Jo software (TreeStar, CA).

Gene reporter assay

The serum responsive element (SRE) activity was determined as described (244). Briefly, NIH-3T3 cells were plated into 24-well plate and pcDNA3.1-N-mycPDZ-RhoGEF was transfected together with pSRE luciferase reporter construct and pCMV-β-gal using Optiman/Lipofectamine system (Invitrogen). Six hours after transfection, the transfectant was removed and replaced with fresh complete culture medium. The cells were starved with 0.5% FCS for 24 hours and stimulated with FCS (10%) and IGF-1 (100 ng/ml) for 6 hours before harvest. Cells were then lysed and luciferase activity was measured (Promega). The actual SRE
activity was normalized by $\beta$-galactosidase activity.

Gene-targeting construction, PDZ-RhoGEF null mouse, and mouse embryonic fibroblasts (MEFs)

The gene-targeting construct was generated using a FRT/loxp based construct, which is used for generating either a conventional targeting construct or a tissue-specific conditional targeting construct depending on the introduction of a specific recombinase respectively. A replacement vector (pSPUC) was generated in which an FRT-flanked neo expression cassette was introduced into an intron between exon 1 and 2 of PDZ-RhoGEF and two loxP sites were engineered to flank the targeted exon 2, which created a null allele of PDZ-RhoGEF when cre recombinase was introduced. The targeting construct was electroporated into embryonic stem cells (ES) from 129J/HSD strain. The homologous recombination and specific integration were confirmed by genomic southern blot. Subsequently, the homologous recombined ES clones were microinjected into blastocysts isolated from C57/BL6 strain and transferred into the 129J/HSD foster mother. The germ line transmission was confirmed by genomic southern blot. Congenic C57BL6 PDZ-RhoGEF knockout strains were obtained by backcrossing the original gene targeted 129J/C57BL6 mixed strains independently for 6 to 10 generations to inbred C57BL6 mice from The Jackson Laboratory. Congenic C57BL6 PDZ-RhoGEF knockout mice and littermate wild type control mice were generated by introducing a cre recombinase under the transcriptional control of a human cytomegalovirus (CMV) promoter (450). The deletion of exon
2 was confirmed by PCR with specific primers, Southern blot with flanking probe, and Western blot with anti-PDZ-RhoGEF (TC-1) antibody. Mouse embryonic fibroblasts (MEFs) were derived from embryos at day 14.5 (E14.5) as passage 0. All the experiments were carried out using early passages of primary mouse embryonic fibroblasts (passage 3) derived from F6 congenic C57BL6 male embryos and the littermate wild-type MEFs were used as controls. All procedures involved in animal studies were in accordance with the guidelines of The University Health Network Laboratory Animal Research Center.

**RBD-binding assay**

*In vivo* RhoA activity was assessed by a modified method as described in the manufacture’s protocol (Upstate). Briefly, serum starved retroviral infected NIH-3T3 or mouse embryonic fibroblasts were stimulated with FCS (10%), IGF-1 (80 ng/ml), LPA/DMSO (20 µM) for 20 minutes and lysed at 4°C in an Mg buffer (25 mM HEPES, pH7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA, 10% glycerol). Lysates were incubated with agarose-conjugated GST fusion Rho binding domain (RBD) of Rhotekin (Upstate) according to the manufacturer’s protocol and followed by four washes with lysis buffer. GTP-bound RhoA associated with GST-RBD was released from beads, and quantified by Western blot analysis using monoclonal antibody against RhoA (Santa Cruz Biotechnology).

**Western and Southern blotting**

Cells were starved 24 hours (retroviral-infected NIH-3T3 cells) or 48 hours (primary
mouse embryonic fibroblasts) before stimulation with FCS (10%), IGF-1 (80 ng/ml), or LPA (20 μM) for 15 minutes. Tissue samples were dissected from fasted animals (14 to 16 hr starvation) at 5 minutes post human insulin (0.75 U/kg of body weight, Novo Nordisk) challenge. Cells and tissues were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml Leupeptin). Equal amounts of proteins were fractionated by NuPAGE (Novex) and analyzed using antibodies specific for phospho-Akt/PKB (S473), PKB/Akt, and cyclin D1 (Cell signaling). Experiments for IRS-1 phosphorylation and protein-protein interaction involved immunoprecipitating total IRS-1 by anti-IRS-1 antibody and immunoblotting with phospho-specific antibodies for tyrosine (Upstate), S632/635 and S612 of IRS-1, and antibodies for IRS-1, p110α (Cell signaling), and p85α (Upstate).

**Growth curve and body fat measurement of animals**

Both wild type and PDZ-RhoGEF-deficient male mice were weaned at 5 weeks of age and placed on normal chow diet (NCD, 5% fat, Harlan) (n = 8) until 17 weeks of age. Animals were fed with high fat diet (HFD, 45% fat, diet 01435, Harlan (n = 8) from 17 weeks up to 32 weeks of age. Body weight was recorded every week for both NCD (n = 8) and HFD (n = 8) fed mice. To measure white adipose tissues, the white adipose tissues from epididymal, retroperitoneal, and inguinal area were dissected from HFD (n = 8) and NCD (n = 4-5) fed male mice, which was normalized by total body weight.
**Glucose homeostasis study**

Fasting glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed on age matched (10 months) wild type (WT) and PDZ-RhoGEF-deficient mice (PDZ-RhoGEF\(^{-/}\)) every 3 weeks after feeding with HFD. For GTT, PDZ-RhoGEF wild-type mice (n = 8) and mutant mice (n = 8) were starved for 16 hours before intraperitoneal challenge with 1 g D-glucose/kg of body weight. Glucose kinetics was measured within two hours after glucose injection. ITT was done in three hour fasted WT (n = 8) and PDZ-RhoGEF\(^{-/}\) mice (n = 8). Human insulin (1 U/kg of body weight, Novo Nordisk) was given to animals by intraperitoneal or intravenous injection. Glucose levels were measured every 15 minutes within one hour after insulin injection. All blood glucose levels were determined from tail venous blood with an automated glucose monitor (Actusoft, Roche).

**Metabolic studies**

Serum was collected from WT (n = 6) and PDZ-RhoGEF\(^{-/}\) mice (n = 8) after 16 hours of starvation for determination of fasting glucose, insulin, triglycerides, and glycerol. Glucose levels were measured using an automated glucose monitor (Actusoft, Roche). Insulin levels were determined by Ultrasensitive Insulin ELISA Kit (Crystal Chem. Inc.) in triplicates. Fasting triglycerides and glycerides were determined by the serum Triglyceride Determination Kit (Sigma-Aldrich). Free fatty acid (FFA) in serum was determined by colorimetric assay (Wako Chemicals). Fasting levels of adiponectin (Acrp30) was determined using Mouse Adiponectin
Immunoassay from R&D system in triplicates and normalized by body weight.

**Indirect calorimetry**

The oxygen consumption rate (VO$_2$) in 9 to 14 month-old male WT and PDZ-RhoGEF$^{/-}$ mice was determined using the Oxymax system (Columbus Instruments).

**Histology of white adipose tissues and liver**

Dissected livers from the *WT* and *PDZ-RhoGEF$^{/-}$* mice were fixed in 10% phosphate buffered-formalin, embedded in paraffin, sectioned in 4 µm slices, and stained with hematoxylin-eosin (H&E).

**Adipocyte size distribution analysis**

Epididymal white adipose tissues were prepared from NCD-fed 28-week old WT and *PDZ-RhoGEF$^{/-}$* male mice and 32-week old *WT* and *PDZ-RhoGEF$^{/-}$* mice with the last 15 weeks on high-fat diet. Paraffin embedded tissues were sectioned in 4-µm-thick sections and stained with H&E. Adipocyte cell size was determined with Image J software (US National Institutes of Health). At least 100 cells were measured from each genotype (n = 6).

**Isolation of adipose tissue-derived stromal cells (ADSCs) from epididymal white adipose tissues**

Epididymal fat pads were dissected from 28-week old wild type and PDZ-RhoGEF$^{/-}$ male mice (n = 2 for each genotype). Fat pads were digested in Kreb’s buffer (Sigma-Aldrich) with collagenase III (250 U/ml, Gibco) at 37°C for one hour. ADSCs were pelleted by
centrifugation and cultured in F12K/DMEM (1:1) with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 55 mM β-mercaptoethanol.

Assays for mitotic clonal expansion, and in vitro adipocyte differentiation

Post-confluent MEFs were subjected to the adipocyte differentiation induction medium (complete medium/1 mM dexamethasone/5 µg/ml insulin/0.5 mM isobutylmethylxanthine (IBMX)) or insulin (5 µg/ml) alone. Cell cycle re-entry and DNA synthesis was determined by [³H]-thymidine incorporation at the indicated times. BrdU labeling was performed to monitor mitotic clonal expansion. MEFs were plated onto glass coverslips and grown to confluence. 15 hours after induction of differentiation, cells were pulse-labeled for 3 hours with BrdU (10 µM) and then transferred to BrdU-free induction medium. 72-hour later, cells were fixed and BrdU positive cells were detected by phycoerythrin-conjugated anti-BrdU antibody (Molecular Probe, Invitrogen) and counter stained with 4′,6-diamidino-2-phenylindole (DAPI). For in vitro differentiation, post-confluent cells were maintained in induction medium for 2 days, and then were fed with complete medium supplement with 5 µg/ml of insulin every other day. At the end of the monitoring period (day 8), cells were fixed with 10% phosphate buffered formalin. Oil droplets were stained with Oil Red-O as described (Tang et al., 2003). Relative lipid content was determined by absorbance at 510 nm by spectrophotometer (Beckman/Coulter, DU800). Post-confluent ADSCs were stimulated with insulin (5 ng/ml) and DNA synthesis was assessed by [³H]-thymidine incorporation at each indicated time point.


**Results**

**PDZ-RhoGEF couples IGF-I to activation of RhoA signaling cascade**

To explore the biological role of PDZ-RhoGEF in mammals, we generated a stable cell line that expresses PDZ-RhoGEF2 under the control of retroviral promoter, long terminal repeat (LTR). The infection efficiency was determined by the percent of GFP positive cells (Figure III-1A) and protein expression was determined by anti-myc antibody (Figure III-1B). Indeed, cells with LTR-driven PDZ-RhoGEF overexpression exhibited growth advantage under optimal conditions, determined by increased cell numbers (Figure III-2A). To explore the potential role of PDZ-RhoGEF downstream of specific signal mediated cell proliferation, serum starved PDZ-RhoGEF-overexpressing and GFP-control cells were stimulated with various mitogens, including fetal calf serum (FCS, 10%), IGF-1 (75 ng/ml), LPA (20 µM), and EGF (75 ng/ml). Cell proliferation was assessed by [³H]-thymidine uptake at 48 hours after mitogen stimulation and cells displayed approximately a 3-fold increased proliferation in PDZ-RhoGEF-overexpressing cells in response to IGF-1 (Figure III-2B). However, there was no difference or subtle difference in response to serum, EGF and LPA (Figure III-2B).

It has been shown that as a Dbl GEF, PDZ-RhoGEF selectively catalyzes GDP/GTP exchange of RhoA small GTPase in response to various extracellular signals, including thrombin, plexin-B, angiotensin II, and LPA (249, 259, 451, 452). Hence, the involvement of PDZ-RhoGEF in coupling IGF-1 signaling to RhoA activation was tested using an *in vivo* Rho-
Figure III-1. Generation of GFP-control and PDZ-RhoGEF-overexpressing NIH-3T3 cell lines

A. The infection efficiency was determined by FACS analysis based on the intensity of GFP. The expression of PDZ-RhoGEF and GFP is driven by retroviral promoter, *long terminal repeats (LTR)*. The GFP-positive cells represented GFP-control and mycPDZ-RhoGEF-GFP-overexpressing cells, respectively. B. PDZ-RhoGEF expression was confirmed using anti-myc antibody.
Figure III-2. PDZ-RhoGEF promotes cell proliferation and is required for IGF-1 mitogenic effects.

A. Increased cell numbers in cells with ectopic expression of PDZ-RhoGEF. Cells were cultured under normal condition (10% FCS) and cell proliferation was determined by counting cell numbers every day for six days. B. Increased $[^3]$H-thymidine uptake by PDZ-RhoGEF-overexpressing cells in response to IGF-1. The serum-starved cells were stimulated with various mitogens as indicated and the mitogenic effect was determined by measuring DNA synthesis. Statistics: mean +/- S.D.
binding (RBD) assay. The level of GTP-bound RhoA was enhanced in PDZ-RhoGEF-overexpressing cells at 20 minutes after addition of IGF-1 but not serum or LPA compared to controls (C and DMSO) (Figure III-3A). These findings suggest that overexpression of PDZ-RhoGEF specifically couples IGF-1 signaling to RhoA activation in promoting cell proliferation.

As major effectors of activated RhoA, Rho kinases, ROCKI/β and ROCKII/α are involved in various RhoA-mediated cellular processes, including stress fiber formation, cell cycle progression, gene expression, as well as cell transformation and invasion (139, 453-458). In the presence of a small molecule inhibitor of Rho kinases, Y27632, the DNA synthesis in PDZ-RhoGEF overexpressing cells was suppressed and was comparable to GFP control cells in response to IGF-1 (Figure III-3B), suggesting that PDZ-RhoGEF selectively activates RhoA mediated Rho kinase activation in promoting cell proliferation in the presence of IGF-1.

**PDZ-RhoGEF enhances cell proliferation through activation of IRS-1/PI3-kinase signaling cascade**

The serine/threonine kinase PKB/Akt has a central role in insulin/IGF-I signaling. Aberrant PKB/Akt activation underlies multiple cellular responses regulated by insulin/IGF-I signaling, including cell proliferation, survival, growth, and cellular metabolism (459). Phosphorylation at serine 473 (S473) of PKB/Akt is required for full PKB/Akt activation, and S473 phosphorylation in PDZ-RhoGEF-overexpressing NIH-3T3 cells was enhanced in response to serum and was greatly increased in response to IGF-1. Consistent with the RhoA activation
Figure III-3. Overexpression of PDZ-RhoGEF is required for IGF-1 mediated RhoA/ROCK activation.
A. RhoA activity was enhanced in PDZ-RhoGEF-overexpressing cells in response to IGF-1, whereas serum and LPA had no effect on RhoA activation. RhoA activation was determined in serum-starved GFP-control and PDZ-RhoGEF-overexpressing cells by Rho-binding domain (RBD) assay at 15 minutes after IGF-1 (80 ng/ml) stimulation. B. Twenty-four-hour pretreatment of ROCK inhibitor Y27632 (5 μM) suppresses PDZ-RhoGEF-dependent cell proliferation in response to IGF-I (80 ng/ml). Cell proliferation was determined as in Figure III-2B. Statistics: mean ± S.D.
data, LPA had no effect on PKB/Akt activation (Figure III-4A). These data suggest that PDZ-RhoGEF is essential for coupling IGF-1 to RhoA GTPase and PKB/Akt activation.

It is known that activated PKB/Akt exerts its function on cell proliferation through multiple downstream substrates, cell cycle regulators, and pro- or anti-apoptotic molecules (459), and RhoA activation has been linked to cell cycle progression (115, 121, 123, 460, 461). Thus, we analyzed the cell cycle dynamics of quiescent state GFP-control and PDZ-RhoGEF-overexpressing cells. The cells were arrested in the G₀ phase with 0.5% serum and triggered into cell cycle re-entry by the addition of IGF-I. IGF-1 stimulation resulted in similar cell cycle entry kinetics in GFP-control and PDZ-RhoGEF-overexpressing cells; however, PDZ-RhoGEF-overexpressing cells displayed robust DNA synthesis in the next cell cycle (24 hours to 48 hours) (Figure III-4B). It has been shown that elevated PDZ-RhoGEF is responsible for serum responsive factor (SRF) mediated gene expression (222). However, by measuring luciferase activity under the control of SRE, we found that overexpression of PDZ-RhoGEF had no effect on SRE-dependent gene expression in mouse fibroblasts upon serum and IGF-1 stimulation (Figure III-4C). These findings suggest that overexpression of PDZ-RhoGEF sensitizes cells to IGF-1 stimulation. Mechanistically, overexpression of PDZ-RhoGEF results in RhoA/ROCK activation and PKB/Akt activation leading to continued cell proliferation in the absence of IGF-1 that is regulated independently from SRE-mediated immediate early gene (IEG) expression.

**PDZ-RhoGEF enhances IGF-1 signaling via Rho-kinases-dependent activation of**
Figure III-4. Enhanced PKB/Akt activation is required for PDZ-RhoGEF overexpression enhanced cell proliferation in response to IGF-1.

A. PKB/Akt activation was increased in PDZ-RhoGEF-overexpressing NIH-3T3 cells in response to IGF-1. PKB/Akt activation was determined by measuring the levels of phosphorylation at S473 at 15 minutes after FCS (10%), IGF-1 (80 ng/ml), and LPA (20 μM) stimulation. B. DNA synthesis was more efficient in PDZ-RhoGEF-overexpressing NIH-3T3 cells upon IGF-1 stimulation. DNA synthesis was determined by [3H]-thymidine uptake at each time point as indicated upon IGF-1 (80 ng/ml) stimulation. C. PDZ-RhoGEF overexpression had no effect on serum response element (SRE)-dependent gene expression in response to FCS (10%) and IGF-1 (80 ng/ml). SRE activity was determined at 6 hours after stimulation and normalized by β-galactosidase activity. Statistics: mean +/- S.D.
**IRS-1/PI3-kinase signaling cascade**

In order to elucidate the physiological function of PDZ-RhoGEFs, we generated a PDZ-RhoGEF-null mouse using a gene targeting strategy. An FRT/LoxP system was employed to generate a PDZ-RhoGEF-deficient mouse. The target construct and deletion strategy generated are shown in Figure III-5. Homologous recombination and specific integration in embryonic stem cells was confirmed by southern blot analysis (Figure III-6A). A PDZ-RhoGEF-null animal was generated after deletion of exon 2 by introducing a cre recombinase under the control of human cytomegalovirus (CMV). Deletion of PDZ-RhoGEF was confirmed by southern and western blot analysis (Figure III-6B, 6C). Although PDZ-RhoGEF is ubiquitously expressed (Figure III-7), homozygous null animals were produced at a Mendelian ratio, and showed no defects in development or fertility, suggesting that PDZ-RhoGEF was dispensable for these processes. Moreover, we further confirmed that loss of PDZ-RhoGEF had no effect on RhoA mediated actin cytoskeleton reorganization, such as stress fiber formation and migration, in mouse embryonic fibroblasts (MEFs) from embryos of PDZ-RhoGEF<sup>−/−</sup> and wild type littermates at embryonic day 14.5 in response to serum, IGF-1, and LPA (Figure III-8).

As expected, PDZ-RhoGEF<sup>−/−</sup> MEFs displayed significant reduction in cell numbers under optimal growth conditions (Figure III-9A). As well, deletion of PDZ-RhoGEF desensitized MEFs in response to serum or IGF-1 but not LPA, which was indicated by a decrease in DNA synthesis (Figure III-9B). PDZ-RhoGEF was essential for RhoA activation in response to IGF-1
Figure III-5. Targeted locus of mouse PDZ-RhoGEF gene (arhgef11).
(i) The 5' genomic region of arhgef11, (ii) the gene-targeting vector, (iii) the targeted allele, and (iv) the mutated allele. Flanking probe (Probe A), neomycin (Neo: Probe B), phosphoglycerol kinase promoter (PGK), cyclization recombinase (Cre), flipase recognition target (FRT), locus X over P1 (loxP), mutant (mt), and wild type (WT), and stop codon (*) are indicated.
Figure III-6. Confirmation of homologous recombination and complete deletion of PDZ-RhoGEF (arhgef11.)

A. Homologous recombination in embryonic stem cells was confirmed by Southern blot analysis. Genomic DNA was digested with EcoRI. An approximately 500 bp DNA fragment (Probe A) at the 5' end of the recombination site was used to detect the targeting vector integration and a neo probe (Probe B) was used to monitor for the number of integration events. B. Following genetic introduction of Cre recombinase, the gene deletion in embryonic mouse fibroblasts (MEFs) derived from day 14.5 embryos (E14.5) was confirmed by Southern blot analysis. C. Loss of PDZ-RhoGEF protein expression in MEFs (E14.5). PDZ-RhoGEF expression was detected by immunoblotting MEF lysates using an anti-PDZ-RhoGEF antibody.

Genotypes: WT (PDZ-RhoGEF+/+), mt (targeted allele), heterozygous mutant allele (PDZ-RhoGEF+/−), and homozygous mutant allele (PDZ-RhoGEF−/−).
Figure III-7. The tissue expression profile of PDZ-RhoGEF and ROCK(s).
PDZ-RhoGEF is ubiquitously expressed in various adult mouse tissues as indicated. Equal amount of protein from tissue lysates were analyzed by antibody specific for PDZ-RhoGEF, ROCKI/β, and ROCKII/α. HT: hypothalamus, Sm. int.: small intestine, EWAT: epididymal white adipose tissue, LN: lymphnode, M: mammary gland (virgin female).
Figure III-8. PDZ-RhoGEF is dispensable for actin stress fiber formation and mitogen-induced cell migration.

A. Stress fiber formation was determined by phalloidin-rhodamine staining at 15 minutes after mitogen stimulation. B. Twenty-four hours after mitogen stimulation, cell migration of WT and PDZ-RhoGEF<sup>−/−</sup> MEFs was determined by the wound-healing assay. C. Six hours after plating, IGF-1 induced migration of cells with the indicated genotype was determined by the transwell migration assay.
Figure III-9. PDZ-RhoGEF is required for primary mouse embryonic fibroblast (MEF) proliferation and RhoA activation in response to IGF-1.
A. MEFs lacking PDZ-RhoGEF exhibited less proliferative activity than wild type MEFs. The cell proliferation was determined by quantifying cell number under normal culture condition (10% FCS). B. Loss of PDZ-RhoGEF impaired MEF proliferation in response to IGF-1 but not LPA. Twenty-four hours after the addition of the indicated factors, [³H]-thymidine incorporation in MEFs was measured. C. Significantly, PDZ-RhoGEF⁻/⁻ MEFs failed to activate RhoA in response to IGF-1. Active form of RhoA (RhoAGTP) in MEFs was determined by RBD assay at 15 minutes after mitogen stimulation. Statistics: mean ±/− S.E.M.; **P < 0.01, versus wild type, (2-tailed Student's t-test).
and serum, but was dispensable for stimulation by LPA (Figure III-9C). To further confirm the function of PDZ-RhoGEF in IGF-1 sensitivity, PKB/Akt activation was tested in the MEFs. While both genotypes displayed IGF-1-dependent PKB/Akt phosphorylation at S473, the level of induction was far lower in PDZ-RhoGEF−/− MEFS (Figure III-10A). Importantly, activation of the IGF-1 receptor, judged by its activation-specific tyrosine phosphorylation of β-subunit, was comparable between the two genotypes (Figure III-10B), indicating that diminution of PKB/Akt phosphorylation in PDZ-RhoGEF−/− MEFS stems from an impaired PDZ-RhoGEF-dependent step downstream of the receptor. Significantly, pretreatment of wild type MEFs with Y27632, a specific inhibitor of the Rho kinases (ROCKs), reduced IGF-1-dependent PKB/Akt phosphorylation to the levels found in PDZ-RhoGEF−/− MEFS (Figure III-10C). Together with the result shown in Figure III-2D, we demonstrated that ROCKs mediate the PDZ-RhoGEF-dependent biological and biochemical outputs in response to IGF-1. ROCKs have been previously implicated in direct phosphorylation of IRS-1 (143, 195, 196, 462). Specifically, ROCKs have been found to modulate insulin/IGF-1 signaling, both positively and negatively, via phosphorylation of IRS-1 at residues S632/635 and S612, respectively (143, 193, 195, 196, 198, 462, 463). We found that IRS-1 S632/635 phosphorylation was greatly reduced in PDZ-RhoGEF−/− MEFS compared to that in wild type MEFS (Figure III-10C). In addition, a decrease in association of IRS-1 with the p85α and p110α subunits of PI3-kinase was observed (Figure III-10C). Nevertheless, IRS-1 S612 phosphorylation in MEFS was not affected by the PDZ-RhoGEF
Figure III-10. PDZ-RhoGEF couples IGF-1 signaling to ROCK-dependent activation of IRS-1/PI3-kinase signaling.

A. IGF-1-mediated PKB/Akt activation was decreased in PDZ-RhoGEF^-/- MEFs and wild type (WT) MEFs in the presence of Rho kinase inhibitor, Y27632. PKB/Akt activation was determined by p-S473 PKB/Akt immunoblotting at 15 minutes after IGF-1 stimulation. Wild type MEFs were treated with Y27632 (20 μM) for 24 hours prior to IGF-1 (80 ng/ml) addition. B. IGF-1 receptor activation is not affected by PDZ-RhoGEF loss. IGF-1 receptor β subunit (IGF-1Rβ) activation was determined by Y1135/1136 phosphorylation by immunoblotting. C. Impaired IRS-1 S632/635, but not S612 phosphorylation, and association with PI3-kinase (left) were observed in PDZ-RhoGEF^-/- MEFs in response to IGF-1. D. IRS-1 S632/635 phosphorylation was reduced in the presence of ROCK inhibitor but not rapamycin. Top: wild type MEFs pretreated with or without Y27632 (20 μM, 24 hrs) or rapamycin (100 nM, 1 hr) prior to IGF-1 stimulation (15 min). Bottom: rapamycin does not affect IRS-1 S632/635 phosphorylation in response to IGF-1 in MEFs. Whole cell lysates were probed with the indicated antibodies.
deletion, indicating that phosphorylation of this residue in MEFs can be uncoupled from that of S632/635 (Figure III-10C). Notably, IGF-1 mediated IRS-1 S632/635 phosphorylation in wild type MEFs was blocked by ROCK inhibitor, Y27632, but was not affected by the mTOR inhibitor, rapamycin (Figure III-10D). Taken together, our results firmly implicate differential IRS-1 phosphorylation as a key mediator of PDZ-RhoGEF effects on downstream signaling.

Reduced adipose tissue size in PDZ-RhoGEF<sup>−/−</sup> mice

As the mice aged, PDZ-RhoGEF<sup>−/−</sup> animals appeared smaller than their wild type counterparts. To further assess a possible effect of PDZ-RhoGEF deletion on mouse growth, an age-matched set of six week-old wild type and PDZ-RhoGEF<sup>−/−</sup> mice from both genders were placed on a normal chow diet (NCD) (5% total calories derived from fat, 200 kcal/kg) for 12 weeks. Throughout the monitoring period, both male ($P = 0.02$) and female ($P = 0.003$) PDZ-RhoGEF<sup>−/−</sup> mice exhibited significantly lower body weight compared to their wild type counterparts (Figure III-11A & 11-B). The differential weight gain was not a result of altered food uptake since animals of both genotypes displayed equal food consumption (Figure III-11C). Upon dissection, the PDZ-RhoGEF<sup>−/−</sup> mice displayed a marked reduction in white adipose tissue (WAT) mass (Figure III-12A). When normalized to overall body weight, epididymal, inguinal, and retroperitoneal fat pads were significantly smaller in PDZ-RhoGEF<sup>−/−</sup> animals (Figure III-12B). Adipose tissue growth results from an increase in both adipocyte cell size and cell number, and occurs as part of normal body growth, as well as during the development of obesity (436-
Figure III-11. PDZ-RhoGEF-deficient mice are leaner and show no difference in food intake.
A & B. Both male (n = 8) and female (n = 18) PDZ-RhoGEF−/− mice exhibited less body weight gain than wild type mice. Body weight under normal chow diet (NCD) was monitored weekly from 6-weeks to 17-weeks of age. C. Loss of PDZ-RhoGEF had no effect on food intake. Food intake per mouse was measured over 15 days (n = 4-5). Statistics: mean ±/− S.E.M.; *P < 0.05, **P < 0.01, versus wild type, (2-tailed Student's t-test).
Figure III-12. Decreased body weight in male PDZ-RhoGEF-null mice is due to reduced adipose tissue mass. 
A. PDZ-RhoGEF<sup>−/−</sup> male mice displayed smaller white adipose tissue (WAT) mass. Representative epididymal (EWAT), retroperitoneal (RWAT), knee (KWAT), and inguinal (IWAT) white adipose tissue depots from 7-month old WT and PDZ-RhoGEF<sup>−/−</sup> male mice fed with NCD. B. Reduced white adipose tissue weight in 7-month old PDZ-RhoGEF<sup>−/−</sup> male mice fed with NCD. The weight of EWAT, RWAT, KWAT, and IWAT was normalized to overall body weight (n = 8). C. Loss of PDZ-RhoGEF had no significant effect on adipose cell size. H&E stained paraffin sections of EWAT from NCD-fed WT and PDZ-RhoGEF<sup>−/−</sup> mice (left) and distribution of adipocyte cell size (right) were presented. At least 100 adipocytes from each genotype was measured and analyzed by Image J software (n = 6). Scale bar: 100 μm. Statistics: mean ± S.E.M., *P < 0.05, **P < 0.01, versus wild type, (2-tailed Student's t-test).
Histological analysis demonstrated that the distribution of adipocyte size of epididymal WAT (small and medium) from wild type and PDZ-RhoGEF−/− animals was not statistically significant under normal conditions (Figure III-12C). Consistent with the lack of PDZ-RhoGEF expression in the skeletal muscle (Figure III-13A), muscle weight was unaffected by the PDZ-RhoGEF loss (Figure III-13B). Finally, judged by a 17.5% to 23% increase in oxygen consumption in PDZ-RhoGEF−/− mice, metabolic rates were significantly increased compared to their wild-type counterparts (P < 0.05) (Figure III-13C). Taken together, the phenotype of PDZ-RhoGEF−/− mice implicates PDZ-RhoGEF as a key factor in regulation of body weight and adipose tissue homeostasis.

**Loss of PDZ-RhoGEF protects from obesity-induced insulin resistance and type II diabetes**

To further investigate the role of PDZ-RhoGEF in the development of diet-induced obesity, a group of 4 month-old PDZ-RhoGEF−/− and wild-type animals were each transferred onto a high fat diet (HFD) (45% total calories derived from fat, 2,025 kcal/kg) for 14 weeks. Both wild type and PDZ-RhoGEF−/− mice from both genders gained weight upon switching to HFD (Figure III-14A and 14B). Nevertheless, wild-type mice maintained a proportionally higher body weight while PDZ-RhoGEF−/− mice had lower body weight despite being fed a HFD, regardless of gender (Figure III-14A and 14B). Wild type mice exhibited considerably larger epididymal and inguinal adipose tissue mass than PDZ-RhoGEF-null animals (Figure III-15A),
Figure III-13. Lack of muscle phenotypes and increased oxygen consumption in PDZ-RhoGEF-knockout mice.

A. PDZ-RhoGEF protein was not detected in wild type skeletal muscle. PDZ-RhoGEF protein expression in the tissues, including brain, EWAT, liver, and skeletal muscle was determined by immunoblotting. B. Loss of PDZ-RhoGEF had no effect on skeletal muscle mass. C. Higher oxygen consumption was observed in PDZ-RhoGEF-/- mice. Oxygen consumption rate (VO_2) of mice was determined via indirect calorimetry during the dark (6 p.m. to 6 a.m.) and light (6 a.m. to 4 p.m.) period (n = 4-7). Statistics: mean +/- S.E.M., *P < 0.05; **P < 0.01, versus wild type, (2-tailed Student's t-test).
Figure III-14. Loss of PDZ-RhoGEF protects mice from high fat diet-induced obesity.
A&B. Both male (n = 8) and female (n = 18) PDZ-RhoGEF<sup>−/−</sup> mice displayed less body weight than wild type mice under high-fat diet (HFD). Body weight of mice was monitored from 18 weeks to 32-week of age (n = 8). C. Representative WT and PDZ-RhoGEF<sup>−/−</sup> mice at 32-week of age following 15-week of HFD feeding. Statistics: mean +/- S.E.M., *P < 0.05, **P < 0.001, versus wild type, (2-tailed Student's t-test).
Figure III-15. Decreased adipose tissue mass is partly due to differential adipocyte cell size distribution between HFD-fed WT and PDZ-RhoGEF^-/- mice.

A. White adipose tissue weight was reduced in male PDZ-RhoGEF^-/- mice under HFD. The weight of EWAT, KWAT, IWAT, and RWAT, respectively, from 32-week old mice after 15 weeks on HFD was normalized to overall body weight (n = 8). B. PDZ-RhoGEF^-/- mice exhibited lower fasting triglyceride than WT mice (n = 9-12). C. PDZ-RhoGEF^-/- mice exhibited more adipocytes in smaller cell-size range, whereas the frequency of adipocytes in the medium cell-size range was significantly higher in wild type mice. H&E stained paraffin sections of EWAT from HFD-fed WT and PDZ-RhoGEF^-/- mice (left) and the distribution of adipocyte cell size (right) were presented. At least 100 adipocyte cells from each genotype were measured and analyzed by Image J software (n = 6). Scale bar: 100 µm. Statistics: mean +/- S.E.M., *P < 0.05, **P < 0.01, versus wild type, (2-tailed Student's t-test).
accompanied by elevated fasting serum triglycerides and glycerides (Figure III-15B).

Histological analysis revealed that epididymal WAT from PDZ-RhoGEF\(^/-\) mice contained a significantly greater proportion of small adipocytes (\(P < 0.05\)) at the expense of midsized adipocytes (\(P < 0.01\)) (Figure III-15C).

Obesity is associated with the development of glucose intolerance and insulin resistance (464). We tested the efficiency of glucose clearance by performing a glucose tolerance test (GTT) following intraperitoneal (IP) glucose injection into male mice after 16 to 18 hours of starvation. When on NCD, animals of both genotypes exhibited comparable glucose tolerance profiles (Figure III-16A). However, despite the maintenance of normal fasting glucose levels in animals of both genotypes after 15-weeks on HFD, wild type male mice displayed impaired glucose clearance (Figure III-16A). These findings indicate that PDZ-RhoGEF loss prevents the development of diet-induced glucose intolerance in male mice. Consistent with the previously noted gender-differences in obesity-related glucose metabolism (465, 466), female mice from both genotypes under the same feeding regimen did not exhibit differences in GTT (Figure III-16B). For these reasons, the subsequent work focused on characterizing the male phenotype in response to insulin. Male PDZ-RhoGEF\(^/-\) animals at this stage maintained full insulin sensitivity as judged by the insulin tolerance test (ITT), which was significantly impaired in wild type mice (Figure III-16C), indicating that PDZ-RhoGEF loss can prevent the development of diet-induced insulin resistance. Consistent with their obese state, wild type mice on HFD, unlike PDZ-
Figure III-16. Lack of PDZ-RhoGEF prevents male mice from high-fat diet induced insulin resistance.

A. Male mice from both genotypes displayed similar glucose clearance efficiency under NCD (17 week). After 15 weeks under HFD, HFD-fed 32-week old male PDZ-RhoGEF<sup>−/−</sup> mice displayed normal glucose tolerance, whereas male wild type mice under same conditions showed impaired glucose tolerance. Glucose tolerance was determined by glucose tolerance test (GTT) on overnight-fasted animals of both genotypes after IP injection with glucose (0.5 g/kg). The levels of glucose clearance efficiency was based on the levels of glucose at indicated time after glucose injection (n = 8). B. HFD-fed 32-week old female mice from both genotypes showed no difference in glucose tolerance. Glucose tolerance was determined as described in (A). C. Male PDZ-RhoGEF<sup>−/−</sup> did not develop insulin resistance determined by insulin tolerance test (ITT). Insulin-mediated glucose clearance (ΔG) was determined at 15 minute intervals after insulin (1 U/kg) was administrated to mice after 15 weeks of HFD feeding through IP injection (n = 8). D. Fasting plasma insulin levels were increased in both 32-week old male WT and PDZ-RhoGEF<sup>−/−</sup> mice on HFD for 15 week (n = 6-8). WT mice exhibited significantly greater levels of fasting plasma insulin. Statistics: mean ± S.E.M., *P < 0.05, ** P < 0.01, versus wild type, (2-tailed Student's t-test).
RhoGEF\(^{-/-}\) animals, displayed elevated circulating insulin levels (Figure III-16D), developed fatty liver contributing to severe hepatic steatosis (Figure III-17A), as well decreased insulin sensitivity in EWAT and liver, determined by activation-specific PKB/Akt S473 phosphorylation (Figure III-17B). Finally, after being on HFD for 6 months, wild type, but not PDZ-RhoGEF\(^{-/-}\) mice, developed chronic hyperglycemia, a hallmark feature of type II diabetes (Figure III-17C). Notably, the serum levels of adiponectin (Acrp30) were significantly higher in PDZ-RhoGEF\(^{-/-}\) mice (Figure III-17D). Taken together, our results reveal an essential role for PDZ-RhoGEF in regulating diet-induced obesity and insulin resistance, ultimately the development of type II diabetes.

**Impaired IRS-1-mediated signaling in EWAT of PDZ-RhoGEF\(^{-/-}\) mice**

To explore the effect of PDZ-RhoGEF deficiency on insulin sensitivity, we tested the activation of insulin signaling pathways in EWAT from our mice. Five minutes following intravenous (IV) injection of insulin into mice on NCD, the EWAT insulin receptors (IR) of both genotypes were activated to comparable levels as shown by Western blotting with activation-specific tyrosine phosphorylation (Y1150/51) (Figure III-18A). Nevertheless, activation of PKB/Akt, measured by the activation-specific phosphorylation at S473 and T308, was significantly lower in EWAT from PDZ-RhoGEF\(^{-/-}\) animals (Figure III-18A), indicating that PDZ-RhoGEF participated in the propagation of insulin signal, acting downstream of the insulin receptor. Interestingly, despite prominent expression in the liver, loss of PDZ-RhoGEF did not
Figure III-17. Deletion of PDZ-RhoGEF protects mice from high fat diet-induced fatty liver (steatosis) and hyperglycemia.

A. PDZ-RhoGEF-null mice were protected from diet-induced fatty liver development. Representative histological liver sections were prepared from WT and PDZ-RhoGEF−/− mice before (NCD) and after switching to HFD for 24 weeks (HFD). Scale bar: 100 μm. B. PDZ-RhoGEF−/− mice maintained normal insulin sensitivity following HFD. Insulin sensitivity was determined by the activation-specific PKB/Akt S473 phosphorylation after immunoblotting protein lysates from EWATs and livers from both genotypes at 15 minutes after IP injection of insulin (1 U/kg). C. Fasting glucose determined before (NCD) and after (HFD). Both genotypes exhibited similar levels of fasting glucose at 17 weeks of age before switch to HFD (n = 6-7). Fasting glucose was significantly higher in 40-week old WT mice than PDZ-RhoGEF-null mice after 24 week on HFD (n = 7-8). D. PDZ-RhoGEF−/− mice exhibited significantly higher plasma adiponectin (Acrp30) levels in relation to body weight compared to WT controls (n = 8-10). Statistics: mean +/- S.E.M., *P < 0.05, versus wild type, (2-tailed Student's t-test).
Figure III-18. PDZ-RhoGEF is required for full activation of insulin signaling cascade in adipose tissue downstream of insulin receptor.

A. S473 phosphorylation of PKB/Akt was reduced, but not phosphorylation of IRβ tyrosine at Y1150/1151 in PDZ-RhoGEF<sup>−/−</sup> EWAT. B & C. Liver and skeletal muscle of PDZ-RhoGEF<sup>−/−</sup> male mice displayed normal insulin sensitivity. EWAT lysates were immunoblotted using indicated antibodies. D. IRS-1 S632/635 phosphorylation and association with PI3-kinase in PDZ-RhoGEF<sup>−/−</sup> EWAT was reduced. IRS-1 was immunoprecipitated with anti-IRS-1 and immunoblotted with the indicated antibodies. EWAT lysates were prepared from WT and PDZ-RhoGEF<sup>−/−</sup> mice 5 minutes (IV) or 15 minutes (IP) after insulin injection.
affect PKB/Akt phosphorylation in response to insulin in this organ (Figure III-18B). Consistent with the lack of expression in the skeletal muscle, PDZ-RhoGEF deletion did not affect PKB/Akt status in this tissue (Figure III-18C). IRS-1 is a multiply phosphorylated protein that acts immediately downstream of the insulin receptor to propagate its signals (467). While the IR-mediated tyrosine phosphorylation of IRS-1 promotes signaling throughput via this adaptor protein, multiple serine/threonine IRS-1 phosphorylation events by various kinases can augment or impede such a response (143, 195, 468, 469). We investigated the phosphorylation of IRS-1 in EWAT from our mice and found significant reduction in S632/635 IRS-1 phosphorylation in animals lacking PDZ-RhoGEF (Figure III-18D). Moreover, in response to insulin, direct association of IRS-1 with the p85α and p110α subunits of PI3K in PDZ-RhoGEF−/− EWAT was reduced (Figure III-18D), indicative of impeded insulin signaling via the IRS-1, consistent with the positive role of IRS-1 S632/635 phosphorylation in promoting insulin signaling (196, 198).

**PDZ-RhoGEF regulates adipocyte differentiation**

Adipose tissue growth results from an increase in adipocyte cell size and cell number through adipogenesis, a process that produces new adipocytes from adipogenic precursor cells or preadipocytes. Adipogenesis is a multi-step process that is regulated by the cross-talk between cell cycle regulators and differentiation factors. During adipogenesis, three proliferative phases that determine the number of adipocytes take place prior to differentiation, a lipid-laden process that accounts for the size of adipocytes {Avram, 2007 #59}. Therefore, it raises the possibility
that the reduced white adipose tissue mass in the absence of PDZ-RhoGEF was due to the impaired adipogenesis. Therefore, we first examined whether loss of PDZ-RhoGEF affects adipogenesis. Similar to preadipocytes, MEFs are capable to differentiate to mature adipocytes.

We examined the impact of PDZ-RhoGEF on adipocyte differentiation of primary E14.5 mouse embryonic fibroblasts (MEFs) derived from PDZ-RhoGEF−/− mice. Terminal adipocyte differentiation of PDZ-RhoGEF−/− MEFs, measured by intracellular lipid staining was greatly reduced compared to their wild type counterparts (Figure III-19A). It is known that insulin/IGF-1 signaling is critically important for adipocyte differentiation (400, 473-478). Moreover, it has been implicated that insulin and IGF-1 has mitogenic effect on the last proliferative phase of adipogenesis, also known as mitotic clonal expansion (13, 431, 472, 479). Together, it suggests that insulin/IGF-1 signaling has a role in adipose tissue growth via regulating the number and the size of adipocytes. Interestingly, under normal food condition, the distribution of adipocytes size was not affected in the absence of PDZ-RhoGEF (Figure III-15B). Using preadipocyte cell lines, the mitotic clonal expansion occurs at 48 hours after induction of differentiation and it is determined by post-confluent mitosis (DNA synthesis), the phase immediately prior to clonal expansion (cell doubling). To determine how PDZ-RhoGEF impacted these processes, post-confluent mitosis and clonal expansion were monitored by [3H]-thymidine uptake and pulse-chase BrdU labeling at 16 hours after induction of differentiation, respectively. PDZ-RhoGEF−/− MEFs displayed greatly diminished DNA synthesis and cell proliferation compared to wild-type
Figure III-19. PDZ-RhoGEF regulates clonal expansion during adipocyte differentiation in vitro.
A. *PDZ-RhoGEF*<sup>+/−</sup> MEFs displayed ineffective adipocyte differentiation. Differentiated cells from each genotype were stained with Oil Red-O for lipid content. Oil Red-O staining was quantified by absorbance at 510 nm. Statistics: mean +/- S.D., **P < 0.01, versus wild type, (2-tailed Student's t-test).
B. Loss of *PDZ-RhoGEF* affected mitotic clonal expansion during adipogenesis. Less BrdU-positive *PDZ-RhoGEF*<sup>+/−</sup> MEFs were detected at 72 hours after induction of adipocyte differentiation (left). *PDZ-RhoGEF*<sup>+/−</sup> MEFs exhibited less [³H]-thymidine incorporation at 16 hours after induction of adipocyte differentiation (right).
C. Lack of PDZ-RhoGEF desensitized EWAT-derived stromal cells (ADSC) to insulin induced DNA synthesis during mitotic clonal expansion.
D. Overexpression of *PDZ-RhoGEF* resulted in increased DNA synthesis in 3T3-L1 cells after induction of adipocyte differentiation. DNA synthesis was determined by [³H]-thymidine incorporation and was measured up to 30 hours after induction of differentiation or addition of insulin. Statistics: mean +/- S.D.
cells (Figure III-19B). To further assess whether PDZ-RhoGEF also regulates insulin sensitivity in adult preadipocyte during mitotic clonal expansion, we examined DNA synthesis in stromal cells derived from adult EWAT (ADSCs) upon insulin stimulation. Similar to the MEFs, PDZ-RhoGEF−/− ADSCs exhibited 2-fold lower thymidine incorporation (Figure III-19C). Finally, to determine if excess PDZ-RhoGEF can promote DNA synthesis during clonal expansion, we generated 3T3-L1 cells stably overexpressing human PDZ-RhoGEF. Unlike control cells, in response to the differentiation mixture, PDZ-RhoGEF-overexpressing cells mounted a robust proliferative response (Figure III-19D). Thus, PDZ- RhoGEF acts as a key regulator of cell proliferation at clonal expansion phase during adipocyte differentiation.

**PDZ-RhoGEF promotes cell survival and transformation via RhoA/ROCK(s)**

Previous work has established that overexpression of wild type or activated PDZ-RhoGEF leads to cell transformation (244). In line with this, PDZ-RhoGEF overexpression led to anchorage-independent growth of NIH-3T3 fibroblasts grown under optimal conditions (Figure III-20A). Of note, a higher percentage of the cell population in S phase was observed in PDZ-RhoGEF-overexpressing cells at resting stage (0 hr) (Figure III-4C), suggesting that the cells with elevated PDZ-RhoGEF were not fully arrested under starvation condition. In accordance with this, PDZ-RhoGEF-overexpressing cells showed 3-fold less serum withdrawal-mediated p21\textsuperscript{CIP1/WAF1} promoter activity than GFP-control cells (Figure III-20B). We also evaluated the growth of PDZ- RhoGEF-overexpressing NIH-3T3 cells in the absence of serum.
Figure III-20. ROCK(s) is required for PDZ-RhoGEF-dependent cell proliferation and transformation.
A. Overexpression of PDZ-RhoGEF resulted in anchorage-independent cell proliferation of NIH-3T3 fibroblasts under normal culture condition (10% FCS). Foci were visualized by crystal violet staining. B. PDZ-RhoGEF-overexpressing NIH-3T3 fibroblasts showed less p21cip1/waf1 promoter activity upon serum withdrawal. The promoter activity is determined by luciferase activity assay. C. ROCK-dependent foci formation was observed in PDZ-RhoGEF-overexpressing NIH-3T3 fibroblasts after 6 days of serum-withdrawal conditions. D. Overexpression of PDZ-RhoGEF promoted cell proliferation of NIH-3T3 fibroblasts in less mitogenic condition (0.5% FCS). Cell number was determined daily. Statistics: mean +/- S.D.
Remarkably, unlike control cells, under conditions of complete serum withdrawal, PDZ-RhoGEF-overexpressing cells formed foci indicative of cell transformation (Figure III-20C). This process was fully dependent on ROCK activity, as the addition of the ROCK inhibitor Y27632 blocked PDZ-RhoGEF-mediated foci formation (Figure III-20C). While control cells overexpressing GFP displayed a minimal increase in cell numbers over time, NIH-3T3 cells overexpressing PDZ-RhoGEF displayed robust proliferation in the absence of serum (Figure III-20D). Six days following serum withdrawal, compared to GFP-overexpressing cells, there was a 10-fold increase in the proportion of PDZ-RhoGEF-overexpressing cells in the S phase of the cell cycle compared to controls (Figure III-21A). Ectopic expression of PDZ-RhoGEF also led to an almost complete resistance to serum-withdrawal-induced apoptosis (Figure III-21A). Finally, and consistent with the linear mechanistic PDZ-RhoGEF/RhoA/ROCK relationship, PDZ-RhoGEF overexpressing cells featured prominent ROCK-dependent S632/635 IRS-1 phosphorylation under low serum condition (0.5% FCS) (Figure III-21B).
Figure III-21. PDZ-RhoGEF mediates cell survival through IRS-1 signaling.
A. *PDZ-RhoGEF*-overexpressing NIH-3T3 fibroblasts were resistant to serum-withdrawal induced apoptosis and maintained cycling status. The proportion of apoptotic cells was determined by annexin-V and 7-AAD staining and was represented by vertical bars (legend on the left). Cells at S phase were determined by PI staining and represented by a line graph (legend on the right). B. ROCK-dependent IRS-1 S632/635 phosphorylation was increased upon *PDZ-RhoGEF* overexpression. Y27632 (20 μM) was added at 48 hours after switching to 0.5% FCS and IRS-1 phosphorylation was determined at 24 hours after addition of Y27632. Whole cell lysates were immunoblotted with the indicated antibodies. Statistics: mean +/- S.D.
Discussion

The twenty members of the Rho family of GTPases are regulated by more than 60 GEFs and 70 GAPs, and are thought to signal downstream through more than 40 effectors (12, 480-482). While such complexity leads to functional redundancy in certain signals, various regulatory inputs display differential specificity toward particular Rho family members and act in a context-dependent manner (249, 258, 452, 483) {Ying, 2009 #1828} {Ying, 2005 #1826}. PDZ-RhoGEF was initially identified as an activator of RhoA acting downstream of GPCRs through association with G\(\alpha_{12/13}\) (222). PDZ-RhoGEF was also found to participate in other signaling pathways and mediate cell migration, axon outgrowth, and chemotaxis (249, 257, 258, 447, 448, 452, 483-485). Using genetic disruption of PDZ-RhoGEF in the mouse, we have shown that PDZ-RhoGEF is dispensable for embryogenesis and a number of known RhoA signaling-mediated cellular processes, including stress fiber formation and cell migration (195, 244, 483, 486-488). Importantly, we have demonstrated a unique role of PDZ-RhoGEF, in coupling insulin/IGF-1 to activation of RhoA GTPase, which enhances cell proliferation and survival in fibroblasts via promoting IRS-1-initiated activation of PI3-kinase-PKB/Akt signaling cascade. Most importantly, PDZ-RhoGEF-dependent signaling appears to be essential for white adipose tissue expansion, which contributes to diet-induced obesity and obesity-related metabolic disorders, including insulin resistance, hyperlipidemia, and type II diabetes.

PDZ-RhoGEF is dispensable for embryogenesis, RhoA-mediated stress fibers and
cell migration

*Drosophila* embryos with homozygous mutation of *DRhoGEF2* die during early embryogenesis, and can be rescued by *PDZ-RhoGEF* (Figure II-3B). This suggests that in the presence of PDZ-RhoGEF can restore DRho1-mediated cell migration and cell shape changes during *Drosophila* embryo development. These findings have directly demonstrated that PDZ-RhoGEF is functionally equivalent to DRhoGEF2. It has been shown that PDZ-RhoGEF interacts with Gα13 and both molecules are co-localized in the neuropil of Purkinje cells. Interestingly, deletion of *Gα13* results in intrauterine death (E9.5-E11.5) with impaired vascular system (378). In contrast to *Gα13*-null mice and *DRhoGEF2* homozygous flies, *PDZ-RhoGEF*-deficient mice appear normal and are born at normal Mendelian ratios. Therefore, we conclude that PDZ-RhoGEF is not required for the Gα13-mediated vascular system homeostasis during embryogenesis. It has been shown that PDZ-RhoGEF and LARG share similar cellular functions regulated by certain ligand/receptor pairs, such as LPA, and semaphorin 4D (249, 258, 261, 451, 452, 489). Although *LARG* knockout mice have not yet been reported, expression of LARG transcript or protein has been found in various tissues and it overlaps with PDZ-RhoGEF (233, 250, 489). Based on the redundant tissue distribution and overlapping cellular functions, we speculate that PDZ-RhoGEF and LARG may compensate for each other during mammalian embryo development.

It has been shown that PDZ-RhoGEF is involved in several activated RhoA small
GTPase-mediated cellular processes, including LPA-mediated stress fiber formation, semaphoring 4D/plexin B-regulated growth cone collapse, adhesion movement and trailing-edge retraction, neurite retraction, SRF/SRE-dependent gene expression, as well as cellular transformation (244, 249, 255, 257, 261, 452, 483). In contrast to these findings, deletion of PDZ-RhoGEF had no effect on RhoA-mediated stress fiber formation and cell migration in response to serum, IGF-1, and LPA (Figure III-8), suggesting that PDZ-RhoGEF specific RhoA activation is dispensable for such processes in primary MEFs. Although interaction of PDZ-RhoGEF with Ga13 enhances its GEF activity, our finding indicates that PDZ-RhoGEF is not essential for Ga13-dependent cell migration (490).

**PDZ-RhoGEF and the regulation of IRS-1**

Further evidence for the function of PDZ-RhoGEF in regulating cell proliferation came from the analysis of PDZ-RhoGEF-deficient MEFs, as well as cells overexpressing PDZ-RhoGEF. Remarkably, NIH-3T3 cells overexpressing PDZ-RhoGEF vigorously proliferate even in the absence of serum, forming foci indicative of transformation. PDZ-RhoGEF-overexpressing cells also display protection from serum-withdrawal-induced apoptosis, likely adding to its transforming potential. The ability of PDZ-RhoGEF to induce proliferation and cause transformation was fully dependent on the activity of ROCK(s), the serine/threonine kinases acting downstream of RhoA, implicating both RhoA and ROCK(s) as the likely effectors of PDZ-RhoGEF function. Taken together, our work establishes PDZ-RhoGEF-dependent
signaling as a potent regulator of cell proliferation. Mechanistically, we have shown that ROCK-dependent IRS-1 phosphorylation at S632/635 is essential for PDZ-RhoGEF-dependent signaling mediated cell proliferation, transformation, and adipose tissue homeostasis.

IRS-1 is a key constituent of insulin/IGF-1-regulated signaling pathways and is subject to extensive regulatory phosphorylation. In addition to tyrosine phosphorylation by activated receptor tyrosine kinases, such as the insulin receptor, which promotes recruitment of SH2 domain-containing downstream effectors, serine/threonine phosphorylation of IRS-1 can affect its signaling capacity both positively and negatively (469, 491). With 182 serine and 60 threonine residues, IRS-1 is a prospective substrate for various serine/threonine kinases (492). Indeed, complex patterns of serine/threonine phosphorylation of IRS-1 reflects the activity of many signaling inputs capable of modulating the insulin/IGF-1 response (493-499). A number of kinases, including ROCKs, JNK (c-Jun NH2-terminal kinase), mTOR (mammalian target of rapamycin), p70S6K, PKCs (protein kinase C ζθλ), IKKβ (inhibitor of nuclear factor kB kinase β), and mitogen activated protein kinase (MAPK), have been found to phosphorylate IRS-1 and are frequently associated with insulin resistance (469, 500-504). The role of IRS-1 S632/635 phosphorylation in mediating insulin sensitivity has been somewhat controversial (196, 505-508). Consistent with our results, ROCKI/β and ROCKII/α mediated phosphorylation of IRS-1 S632/635 were required for promoting insulin-mediated glucose uptake in adipocytes and mouse skeletal muscle, respectively (196, 198). Interestingly, S632/635 of IRS-1 were found to be
phosphorylated together with S307 in p70S6k-dependent manner as part of a rapamycin-sensitive feedback loop upon prolonged exposure to insulin or under HFD, which attenuates insulin signaling (468, 507). Importantly, both IRS-1 S307 and S632/635 phosphorylation were significantly reduced in the fat of mice lacking p70S6K1 or in HeLa cells with their p70S6K1 knocked down after prolonged insulin treatment (468). However, S632/635 of IRS-1 does not appear to a predicted in vivo substrate for p70S6K (499). It is possible that insulin/IGF-1 signaling chronologically regulates phosphorylation of IRS-1 at S307 and S632/635 through distinct mechanisms, reflecting the parallel inputs to IRS-1, with the activating signals via ROCKs and the inhibitory ones via p70S6K1.

The specific role of PDZ-RhoGEF-dependent signaling in adipose tissue expansion

MEFs generated from germline-deletion of Arhgap5, the genetic locus of p190-B RhoGAP displayed a reduction in cell size. Moreover, p190-B RhoGAP-null MEFs failed to undergo adipocyte differentiation, while myogenesis was promoted (143). Consistent with the lack of PDZ-RhoGEF expression in muscle, deletion of PDZ-RhoGEF did not affect MEF size and had no impact on muscle development (Figure III-13). Nevertheless, PDZ-RhoGEF deficiency led to a reduction of committed preadipocyte proliferation, but not their terminal differentiation (Figure III-19). Considering that both p190-B RhoGAP and PDZ-RhoGEF have been found to regulate RhoA, the fact that disruption of these genes does not result in opposing phenotypes may be surprising. Nevertheless, the absence of PDZ-RhoGEF expression in the
muscle, combined with a likely impact of p190-B RhoGAP on the early cell fate determination of mesenchymal precursors, highlights the importance of RhoA signaling at various stages of development of mesenchymal tissues (142, 143). In this context, the specific action of various RhoGEFs could have a distinct impact on adipocyte differentiation. Indeed, in the C2C12 myoblast system, another RhoGEF, GEFT, was found to induce myogenesis while it inhibited adipogenesis of 3T3-L1 preadipocytes (509). Uncoupling of IRS-1 S632/635 and S612 phosphorylation in MEFs upon disruption of PDZ-RhoGEF (Figure III-10C) may indicate another manifestation of the mechanism of achieving specificity in RhoA signaling. Intriguingly, PDZ-RhoGEF appears capable of directing ROCK activity towards IRS-1 S632/635 in fibroblasts and adipose tissue, whereas other GEFs may participate in modulating its activity towards S612 in different tissues. Alternatively, the distinct RhoGEFs may differentially activate ROCK family members, which in turn may harbor subtle, but important differences in specificity towards IRS-1.

Further adding to the complexity of the physiological insulin response, unlike the ubiquitous p70S6K1, PDZ-RhoGEF is not expressed in all cells and tissues. Importantly, muscle, a key insulin target tissue, does not express PDZ-RhoGEF (Figure III-13), whereas the liver, another insulin target tissue where PDZ-RhoGEF is expressed, PDZ-RhoGEF deletion failed to affect PKB/Akt phosphorylation, a measure of insulin signaling throughput (Figure III-18B, III-18C). Such a differential effect might be due to the tissue-specific distribution of downstream
effectors, such as ROCK(s). Mice lacking of ROCKI/β and ROCKII/α displayed distinct phenotypes, despite the high similarity in their kinase domains (183, 487, 488, 510). Although it has been suggested that both ROCKI/β and ROCKII/α transcripts are ubiquitously expressed (181, 183), the protein expression profile in peripheral insulin target tissues is distinct. ROCKI/β is expressed in liver, skeletal muscle, and liver, whereas, the expression of ROCKII/α is only found in white adipose tissue (Figure III-7B). Consistent with its expression profile, ROCKII/α enhances insulin mediated glucose transport in 3T3-L1 adipocytes through phosphorylation of IRS-1 S632/635 (196). Surprisingly, regardless of its expression pattern, ROCKI/β has a major effect on glucose transport in skeletal muscle but has no influence on adipose tissue and liver mass (198). In this context, it is important to note that muscle is the major insulin-stimulated glucose-disposal tissue. Therefore, the tissue-specific expression of ROCKs and Rho GEFs may lead to the differential phenotype resulting from deletion of PDZ-RhoGEF and ROCKI/β, respectively. Nevertheless, these findings demonstrate that S632/635 of IRS-1 is the common substrate for both ROCKs and that phosphorylation of IRS-1 S632/635 by ROCKs enhances insulin action in adipocytes and skeletal muscle.

In contrast to p70S6K knockout mice, there is no distinct cell size difference observed in PDZ-RhoGEF-null and wild type mice under normal condition, however, we have shown that PDZ-RhoGEF is required for hormone induced mitotic clonal expansion that is required for adipocyte differentiation. From the ex vivo differentiation assay, inefficient adipogenesis from
PDZ-RhoGEF−/− MEFs was observed, which correlated with reduced adipose tissue observed in PDZ-RhoGEF−/− mice. Significantly, global or tissue-specific deletion of components within the insulin/IGF-1 signaling pathway, such as the insulin receptor, IRS-1/2, PI3-kinase, p70S6K, and PKBα/Akt1, display a primary effect on adipose tissue mass due to reduction in adipocyte differentiation or adipocyte cell size (396, 468, 473, 474, 478, 511). These findings are a result of the distinct impact of individual insulin signaling components on specific steps of adipocyte/adipose tissue differentiation, which determines the tissue mass. In line with this, our work revealed an exclusive role for PDZ-RhoGEF in mediating insulin/IGF-1-dependent mitotic clonal expansion of adipocyte precursors localized at stromal-vascular fraction of adipose tissue, as well as MEFs. This proliferative phase not only defines the total number of adipocytes, but is also obligate for terminal differentiation, and as such is a key determinant of adipose tissue expansion (512, 513). Mechanistically, adipose tissue mass is regulated distinctly by p70S6K and PDZ-RhoGEF downstream of the insulin receptor. However, it is likely that chronic activation of both pathways results in similar pathological outcome – diet induced obesity and insulin resistance. Conversely, lacking one or the other protects.

Interestingly, under high-fat conditions, PDZ-RhoGEF-null adipose tissue expanded greater than wild type adipose tissue and PDZ-RhoGEF-null mice remained metabolically healthy. These observations imply that PDZ-RhoGEF−/− adipose tissue has higher expandability through increases in newly differentiated adipocytes that are smaller in size. It has been
suggested by pharmacological and genetic evidence that increasing the number of adipocytes accommodates caloric excess and improves insulin sensitivity (514-516). We thus hypothesize that increases in adipose tissue mass by PDZ-RhoGEF-dependent signaling limits caloric storage due to loss of cell expendability when nutrients are overloaded.

**PDZ-RhoGEF and human disease**

Several genome-wide studies have identified genomic heterogeneity at chromosome 1q21-q24 in type II diabetes in various populations (253, 517). More recently, genotyping of multiple single nucleotide polymorphisms within this region has identified variations within the *PDZ-RhoGEF (ARHGEF11)* locus, including the non-synonymous SNP R1467H, as significantly associated with type II diabetes in Pima Indian, Old Order Amish, and German Caucasians (276-278). Interestingly, these SNPs were also associated with differential glucose area under the curve (AUC) during a 3-hour glucose tolerance test (GTT) in healthy individuals, indicative of non-pathological but differential glucose clearance in individuals carrying these polymorphisms, even in the absence of diabetes (276). An independent epidemiological study has found PDZ-RhoGEF polymorphisms associated with a decreased risk of lung cancer in Mexican-American men but not women (279). Interestingly, the non-synonymous SNP S1416G of PDZ-RhoGEF appears to synergize with polymorphisms in the p53 and cyclin D1 genes that are associated with an increase in cancer risk {Betticher, 1996 #2360} {Wu, 2002 #402} {Gu, 2006 #1683}. 
It is noteworthy that both SNPs, the R1467H found in the Pima Indian population and the S1416G found in the Mexican-American population, map to the C-terminus of PDZ-RhoGEF. Deletions of this region have been previously found to result in potent activation of PDZ-RhoGEF by preventing inhibitory homodimerization, as well as heterodimerization with related RhoGEFs LARG and p115RhoGEF (244). Thus, PDZ-RhoGEF SNPs associated with diabetes and cancer may lead to differential guanine nucleotide exchange activity of PDZ-RhoGEF and underlie their association with various pathological states. Based on our data, the prediction is that both SNPs may represent gain-of-function variants. A systematic analysis of the GEF activity of PDZ-RhoGEF variants combined with functional reconstitution of PDZ-RhoGEF−/− cells and mice will be needed to provide further insight into this possibility.

Considering the large number of upstream regulators, Rho GEFs, and multiple downstream effectors, investigation of the molecular network surrounding RhoA and governing its biological function represents a major challenge in biological research. Despite such complexity, genetic disruption of PDZ-RhoGEF uncovered an important and previously unrecognized role of RhoA signaling in regulation of body fat mass and glucose homeostasis. Further work focusing on this aspect of PDZ-RhoGEF may lead to therapeutic opportunities in obesity-related diseases.
Chapter IV

Discussion and Perspectives
Discussion

Insulin/IGF-1 signaling regulates various physiological processes, including cell and organism growth, development, aging, and metabolism mainly acting through IRS and activating major signaling cascades, including Ras-MAPK, PI3-kinase-PKB/Akt pathway, and Cbl/CAP pathway (491) (Figure IV-1). Deregulation of insulin/IGF-1 signaling leads to disease development (415, 421, 518-522). Accumulating evidence points to the existence of an intricate signaling network sensitive to many extracellular signals that modulates the biological output of insulin/IGF-1 signaling (491). Various levels of crosstalk between the insulin/IGF cascades and other signaling pathways, including those elicited by inflammation, stress, and nutrient-sensing, have been documented (523-526). In this study, we have discovered and characterized a conserved role for RhoA signaling as part of insulin/IGF-1 signaling regulatory network in Drosophila and mammals. Using a genetic screen, we have identified a DRho1-specific Rho GEF, DRhoGEF2, which interacts with PTEN/PI3-kinase signaling during Drosophila eye development. DRhoGEF2 and its mammalian ortholog, PDZ-RhoGEF, displayed conserved biological and biochemical functions in controlling neuronal progenitor cell survival in Drosophila through regulation of dPKB/dAkt signaling and photoreceptor terminal differentiation. Although the mechanistic aspects of how DRhoGEF2/PDZ-RhoGEF impacts on PTEN/PI3-kinase signaling remain to be fully elucidated, our work in mammalian model system indicates that PDZ-RhoGEF-dependent signaling is required for maximizing the response of
Figure IV-1. Insulin/IGF-1 signaling.
Upon insulin or IGF-1 binding, insulin receptor (IR) and IGF-1 receptor (IGF-1R) undergo autophosphorylation at several tyrosine residues at cytoplasmic β-subunits. Autophosphorylated (or activated) IR or IGF-1R acts as a tyrosine kinase to further phosphorylate various SH2 domain-containing adaptor molecules, such as insulin receptor substrate (IRS) proteins. Tyrosine phosphorylated adaptor molecules interact with signaling molecules via their SH2-domains, resulting in a diverse series of signal transductions, including activation of PI3-kinase-PKB/Akt, Ras/MAPK, and Cbl/CAP pathways. Together, these signaling pathways work in concert to coordinate various cellular processes, such as vesicle trafficking, protein synthesis, gene expression, enzyme activation and inactivation, which contributes to insulin/IGF-1 signaling-mediated physiological homeostasis, such as cell growth, differentiation, and metabolism.
cells to insulin/IGF-1 stimulation. Through the regulation of IRS-1/PI3-kinase signaling cascade downstream of insulin/IGF-1 receptor, PDZ-RhoGEF was shown to govern fibroblast proliferation *in vitro* and the development of adipose tissue expansion *in vivo*. Upon exposure to excess nutrients, such as high-fat diet, mice deficient for PDZ-RhoGEF failed to developed pathologic traits, obesity and type II diabetes.

**Biology of DRhoGEF2**

Accumulating evidence has shown that DRhoGEF2 regulates cell shape changes that are essential for morphogenesis during embryogenesis as well eye wing development (141, 234, 235, 337, 527). Mechanistically, DRok and Dia mediate recruitment of cortical myosin II and acto-myosin contraction for gastrulation, epithelia folding, and segmentation during embryogenesis (336-338). However, the mechanistic effect of DRhoGEF2/DRh1 on retinal elongation during eye development is not clear. In this study, we established a mechanistic interplay between the DRhoGEF2 signaling cascade and dPKB/dAkt activation, thus further suggesting that DRhoGEF2 signaling affects the insulin-like signaling pathway during *Drosophila* eye development.

DRhoGEF2 contains multiple functional domains and each of these domains may play a role in protein-protein or protein-lipid interactions, suggesting that other signaling molecules may contribute to DRhoGEF2-mediated biological and biochemical outputs. Although a physical interaction between DRhoGEF2 and other signaling molecules has not yet been documentated in
Drosophila, the interaction between mammalian RGS-RhoGEFs with other signaling molecules, such as cell surface receptors (IGF-1R, plexin B, and LPAR1/2), Gα12/13, and scaffolding proteins has been reported. This is essential for mammalian RGS-RhoGEFs mediated RhoA activation and their biological functions (253, 255, 272, 273, 279, 280, 288, 289, 560). Therefore, it raises the possibility that the association of DRhoGEF2 with other signaling molecules may have a major impact on the guanine nucleotide exchange activity of DRhoGEF2 and DRhoGEF2’s biological function. Genetic studies have shown that Drosophila eye development is under spatio-temporal control by single transduction pathways and transcription factors (559). Therefore, in this studies, the observed role of DRhoGEF2 on eye development is tightly linked to a specific developmental signaling pathway at a particular stage during Drosophila eye development. Interestingly, overexpression of DRho1 or DRok-cat in differentiated neuronal cells resulted a rough eye phenotype due to impaired differentiation of both photoreceptor and pigment cells (397, 399). Importantly, cell fate determination at 3rd instar larval stage was not affected when DRho1 or DRok-cat was overexpressed. However, it has been shown that DRho1 is required for Wnt/Frizzled/Dishelleved mediated planar cell polarity during patterning formation at 3rd instar larvae (561). These findings suggest that the effect of Rho signaling on Drosophila eye development is signal and/or cell context dependent. Therefore, further exploring the role of DRhoGEF2 downstream of specific signals may shed the light of cell-context-dependent effect on dPKB/dAkt activation during Drosophila eye development.
In addition, heterozygous DRhoGEF2 has a significant effect on body weight control (Figure IV-2), suggesting that DRhoGEF2 signaling may also participate in physiological processes other than embryogenesis and eye development in *Drosophila*. It will be interesting to elucidate the role of DRhoGEF2 as a signaling integrator beyond Gα-protein coupled signaling in eye development and body weight control.

**PDZ-RhoGEF, a regulator of mammalian adipose tissue development**

In an obese state, adipose tissue becomes dysfunctional, leading to the accumulation of lipid in specific organs and triggers qualitative and/or quantitative changes in adipokine productions. Consequently, obesity leads to the development of metabolic disorders, such as insulin resistance, type II diabetes, cardiovascular disease, and increased risk of cancers, thus increasing morbidity and mortality in humans (464, 528-530). Therefore, understanding the molecular mechanisms involved in adipose tissue development is important for the intervention of obesity and its related disorders.

**Adipose tissue development**

When energy intake is greater than energy expenditure, healthy adipose tissue is able to expand, i.e., increase in adipose tissue mass. To accomplish the storage of surplus triglycerides, the available mature adipocytes can either increase in size (hypertrophy) or increase in number (hyperplasia) by recruiting preadipocytes to differentiate into adipocytes (436, 437, 440, 470). Adipocyte hyperplasia is an essential mechanism for storing excess lipids during the prepubertal
Figure IV-2. The effect of DRhoGEF2 on body weight.
The body weight of male flies was determined at 3 days after eclosion and followed up to day 12 after eclosion (n = 125; 25 flies/vial). DRhoGEF2^{04291} and DRhoGEF2^{5w18} resulted in decreased body weight significantly. Statistics: mean +/- S.E.M.; *P < 0.05; **P < 0.01, versus wild type, (2 tailed Student's t-test).
age, while the increase in mature adipocyte size is predominately seen in adults (438, 471, 531-533).

Under normal food conditions, reduced fat mass in *PDZ-RhoGEF*-deficient mice at 16-weeks of age was not due to reduced adipocyte cell size or food-intake. Nevertheless, we have shown that PDZ-RhoGEF-dependent signaling was found to be essential for mitotic clonal expansion, an early phase of adipogenesis that determines the number of mature adipocytes (472) (Figure IV-3). It suggests that PDZ-RhoGEF has a role in regulating the number of adipocytes. Adipocyte differentiation is tightly orchestrated by a temporally regulated set of gene-expression events, such as three isoforms of CCAAT/enhancer-binding proteins (C/EBPα, β, and δ), and nuclear receptor proteins, peroxisome proliferator-activated receptors (PPAR α, β, δ, and γ). Interestingly, *PDZ-RhoGEF*-null mice are similar to mice that lack one allele of PPARγ (534), mice with adipose tissue-specific deletion of PPARγ (535), and mice lacking C/EBPβ and C/EBPδ (536). Importantly, the insulin/IGF-1 signaling cascade has marked effects on adipogenesis through upregulating the expression of C/EBPα and PPARγ (537-541). We postulate that PDZ-RhoGEF-dependent signaling may regulate C/EBPβ and/or C/EBPδ that is required for C/EBPα and PPARγ expression. Alternatively, PDZ-RhoGEF-dependent signaling may have an effect on the transcriptional activity of PPARγ.

In addition to the clonal expansion of committed preadipocytes, it has been suggested that adipocyte number is also determined by the number of precursor cells, including mesenchymal
Figure IV-3. The cell-autonomous and non-autonomous roles of PDZ-RhoGEF-dependent signaling in insulin/IGF signaling-mediated physiological processes.

(A). PDZ-RhoGEF is required to maximize the mitogenic effect of insulin/IGF signaling on fibroblast proliferation and preadipocyte clonal expansion, a process for increasing the committed preadipocyte population. PDZ-RhoGEF mediates adipose tissue expansion possibly through RhoA/ROCKs-dependent activation of IRS-1/PI3-kinase signaling. (B). When energy intake is greater than energy expenditure, adipose tissue expands to accommodate the storage of excess energy or triglycerides. Under high-fat condition, adipocytes expand in size and develop hypertrophic obesity. In the obese state, adipose tissue becomes dysfunctional and triggers chronic inflammation, which leads to the development of metabolic syndromes. Obesity and its related metabolic syndromes, particularly hyperinsulinaemia, have been tightly linked to poor prognosis and outcome of cancers.
stem cells and preadipocytes (472, 479). Significantly, the number of adipocytes from wild type and PPARγ heterozygous mice under normal condition are comparable, suggesting that PPARγ is necessary for the differentiation but not required for control of adipocyte numbers (534). This difference between PDZ-RhoGEF and PPARγ implies that loss of PDZ-RhoGEF may have additional effects on adipose tissue mass. Indeed, PDZ-RhoGEF−/− preadipocytes derived from the stromal-vascular fraction of white adipose tissue displayed reduced mitogenic response to insulin, suggesting that PDZ-RhoGEF may also play a role in insulin-mediated preadipocyte proliferation (Figure III-19C).

**HFD-induced hypertrophic obesity**

Under the high-fat condition, adipocyte cell size of PDZ-RhoGEF-null mice was uniformly smaller, whereas the adipose tissue from wild type mice exhibited a significant higher number of larger adipocytes. Similarly, the PPARγ−/− adipocytes were smaller than wild type under HFD and these mice were protected from developing HFD-induced hypertrophic obesity. In addition, no liver steatosis was observed (534, 535). In line with this, PDZ-RhoGEF displayed similar effects in HFD-induced hypertrophic obesity and hepatosteatosis as PPARγ. Hypertrophic obesity is accompanied by inflammation, impaired adipogenesis, and insulin resistance, which leads to lipotoxicity, i.e., ectopic lipid accumulation in organs, such as the liver, muscle, heart, and pancreas (542-545). Therefore, PDZ-RhoGEF signaling contributes to hypertrophic obesity-induced abnormality due to its role in regulating adipose tissue
development (Figure IV-3).

**PDZ-RhoGEF and risk of cancer**

Although there is no direct evidence to link the gain-of-function of PDZ-RhoGEF-dependent signaling to tumor development, we have shown in this study that elevation of PDZ-RhoGEF expression in immortalized fibroblasts leads to anchorage-independent proliferation and cellular transformation. Interestingly, elevated PDZ-RhoGEF-dependent signaling results in mitogen-independent survival and cell proliferation, leading to fibroblast transformation. This suggests that overexpression or hyperactivation of PDZ-RhoGEF signaling provides a self-sufficient growth signal through activation of IRS-1/PI3-kinase signaling. Identification of a nonsynonymous single-nucleotide polymorphism S1416G in PDZ-RhoGEF has directly linked PDZ-RhoGEF to the risk of cancer (279). Accumulating evidence has shown that obesity is linked to worse cancer outcome (546-549). The underlying mechanism may involve obesity-associated hyperinsulinemia [Becker, 2009 #4056], growth hormone, IGF-1, levels of endogenous sex steroids, adipose hormones, and/or cytokines [Nomura, #4054] {Park, #4055}(520, 550, 551). Thus, PDZ-RhoGEF may have an indirect role in affecting cancer risk through the regulation of adiposity and whole body metabolism. It remains to be determined whether PDZ-RhoGEF-dependent signaling has autonomous and non-autonomous effects on cancer development and progression (FigureIV-3).

**PDZ-RhoGEF and life span**
Implication from adiposity

In *Drosophila* and mammals, reduction of insulin/IGF-1 signaling and caloric restriction leads to extension of life span (403, 552-557). These findings suggest that life span can be extended when body weight and/or insulin/IGF-1 signaling are reduced. FIRKO mice featuring fat-specific deletion of IR are the first animal model to directly demonstrate the direct effect of insulin signaling on life span due to fat mass changes (392, 396). Interestingly, these are comparable phenotypic characteristics as those found in healthy human centenarians (558, 559). Significantly, *PDZ-RhoGEF*-deficient mice exhibit comparable phenotypes as FIRKO mice and may also have extended life span due to decreased adiposity and body weight. Furthermore, DRhoGEF2 heterozygosity-driven body weight reduction may also prolong *Drosophila*’s life span.

Implications from hair graying

Interestingly, the hair of *PDZ-RhoGEF*-null inbred C57/BL6 mice turned gray around 16 to 20 weeks of age while this was not observed in wild type mice at the same age (Figure IV-4). Hair graying is one of the signs of human aging and is caused by progressive loss of pigmentation from growing hair shafts and it is thought to arise due to a loss of melanocyte stem cells (MSCs) (560). The bulge region of the hair follicle contains a reservoir of epithelial stem cells and undifferentiated MSCs (561). With the initiation of a new anagen, the active phase of the hair follicle cycle, quiescent MSCs proliferate and give rise to melanocyte progenitor cells.
Figure IV-4. Hair graying in PDZ-RhoGEF-deficient mice.
The images represent the appearance of natural hair graying from a year-old wild type and PDZ-
RhoGEF<sup>−/−</sup> male mice.
Thus, hair graying seen in PDZ-RhoGEF-null mice may be due to ineffective MSC proliferation at anagen, leading to reduction of melanin abundance. Is the hair-graying phenotype due to PDZ-RhoGEF-deficiency a sign for premature aging or a loss of survival signaling for melanocyte stem cell population? It will be interesting to further determine the effect of PDZ-RhoGEF on mammalian aging and its role in adult stem cell maintenance.

**PDZ-RhoGEF, as a genetic modifier of PTEN – the evidence from knockout mice**

DRhoGEF2 was first revealed as a genetic modifier of dPTEN. Establishing the interplay between a DRhoGEF2 signaling cascade and dPKB/dAkt activation has further supported this finding. The salvage of *Drosophila* embryonic lethality elicited by DRhoGEF2-null mutation by human *PDZ-RhoGEF* overexpression has strongly supported the view that PDZ-RhoGEF, as a part of Gα-coupled receptor signaling, restores signal transductions and biological functions mediated by DRhoGEF2. Subsequently, the analysis of *PDZ-RhoGEF* knockout mice has disclosed a significant role of a PDZ-RhoGEF-mediated signaling cascade in coupling insulin/IGF-1 signaling to PKB/Akt activation.

Whether PDZ-RhoGEF, as the mammalian ortholog of DRhoGEF2, also interacts with PTEN genetically is not known. Remarkably, half gene dosage of PDZ-RhoGEF expression extended the life span of PTEN heterozygous mice (Figure IV-5). This finding has not only established that the interaction between PDZ-RhoGEF and PTEN is evolutionarily conserved in *Drosophila* and mice, but has also ascertained that PDZ-RhoGEF-mediated oncogenic input
Figure IV-5. PDZ-RhoGEF contributes to PTEN-haploinsufficiency induced tumorigenesis. A Kaplan-Meier Plot demonstrated that the survival of PDZ-RhoGEF<sup>+/−</sup>;PTEN<sup>+/−</sup> mice (n = 22) was markedly increased compared to that of PDZ-RhoGEF<sup>+/+</sup>;PTEN<sup>+/−</sup> mice (n = 4). Statistics: ***P < 0.001, versus wild type, (chi-square).
contributes to tumorigenesis elicited by PTEN mutation. Thus far, DRhoGEF2 and PDZ-RhoGEF genetically interact with PTEN in Drosophila and mice. This underscores the role of PDZ-RhoGEF as part of the insulin/IGF-1 signaling regulatory network.

**Future perspectives**

Several SNPs associated with the risk of cancer and type II diabetes have been identified in the coding and non-coding regions of the PDZ-RhoGEF genomic locus (ARHGEF11). Three SNPs, S1456G, R1467H, and S1416G have been found within exon 39 (276-279) and two other SNPs are located in intron 2 and intron 8 (276). These SNPs may regulate homo- and heterodimer oligomerization between PDZ-RhoGEF and its mammalian homolog, LARG and p115RhoG and/or may possibly generate alternative forms of PDZ-RhoGEF (244). Generation of mouse models with genomic knock-in of these identified SNP mutations may further elucidate how PDZ-RhoGEF contributes to the etiology of obesity, type II diabetes, and cancer. Furthermore, EMS-induced DRhoGEF2 mutant (DRhoGEF2<sup>3w18</sup>) exhibited significantly less body weight compared to P-element mutant (DRhoGEF2<sup>604296</sup>) (Figure IV-2), suggesting that the mutant allele of DRhoGEF2<sup>3w18</sup> may have a dominant-negative effect on the wild type allele. It will be interesting to identify the point mutation and test it.

We have shown in this study that PDZ-RhoGEF signaling has a significant impact on obesity and obesity-related health issues. Several in vitro studies have shown that similar to Vav, the activity of PDZ-RhoGEF is regulated through inter- and intra-molecular auto-inhibition (13,
Moreover, the specific interaction between RhoA switch region and PDZ-RhoGEF DH domain has been revealed by structure analysis (240, 264, 266). These findings together with the normal development found in PDZ-RhoGEF-deficient mice suggest that PDZ-RhoGEF can be considered as the therapeutic target for disease intervention.
Concluding remarks

A combined approach utilizing a genetic modifier screen in *Drosophila* and subsequent genetic and biochemical analyses in mice have identified the genetic interaction between *PDZ-RhoGEF* and *PTEN*. The significance of such interaction is exemplified by the delay in tumor development in PTEN\(^{+/−}\) animals upon deletion of one copy of PDZ-RhoGEF. Considering that obesity and its related metabolic disorders are associated with cancers, the knowledge and reagents generated by this study constitute a foundation for further characterization of PDZ-RhoGEF in various disease states.
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