3-21-2014

Novel Insights into the Mechanisms of Regulation of Tyrosine Kinase Receptors by Ras Interference 1

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DOI: 10.25148/etd.FI14040866

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

NOVEL INSIGHTS INTO THE MECHANISMS OF REGULATION OF TYROSINE
KINASE RECEPTORS BY RAS INTERFERENCE 1

A dissertation submitted in partial fulfillment of the
requirements for the degree of
DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Adriana Galvis

2014

To: Dean Kenneth G. Furton
College of Arts and Sciences

This dissertation, written by Adriana Galvis, and entitled Novel Insights into the Mechanisms of Regulation of Tyrosine Kinase Receptors by Ras interference 1, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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The dissertation of Adriana Galvis is approved.

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College of Arts and Sciences

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University Graduate School

Florida International University, 2014

DEDICATION

I want to dedicate this dissertation, and everything it represents, to my family. To my husband, my parents, and my siblings. I can't thank you enough for the love and support; you made this journey a lot easier. Thanks for helping me make this dream come true.

Quiero dedicarle esta tesis, y todo lo que representa, a mi familia. A mi esposo, mis papas y mis hermanos. No tengo como agradecerles por todo el amor y el apoyo; ustedes hicieron de este, un camino mucho mas facil de recorrer. Muchas gracias por ayudarme a hacer este sueño realidad.

ACKNOWLEDGMENTS

I would like to thank Dr. Alejandro Barbieri for his mentorship and support during the past 8 years.

To Dr. Lidia Kos, Dr. Ophelia Weeks, Dr. John Makemson and Dr. Fenfei Leng for their guidance and input on this project.

To Adriana Marcano, Nathalie Rivero, and Dr. Horacio Priestap (RIP) for their help on specific areas of my research.

To former and current members of Barbieri's lab for their generosity and feedback.

To my friends, especially Monica Isola, Nicole Fresard, Ana Paula Benaduce and Monica Rodriguez for encouraging me throughout this journey.

To the MBRS RISE program, to Florida International University and their staff, especially to Dr. Mo Donelly, for their sponsorship and support.

ABSTRACT OF THE DISSERTATION
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by

Adriana Galvis

Florida International University, 2014

Miami, Florida

Professor Alejandro Barbieri, Major Professor

Receptor-tyrosine kinases (RTKs) are membrane bound receptors characterized by their intrinsic kinase activity. RTK activities play an essential role in several human diseases, including cancer, diabetes and neurodegenerative diseases. RTK activities have been regulated by the expression or silencing of several genes as well as by the utilization of small molecules.

Ras Interference 1 (Rin1) is a multifunctional protein that becomes associated with activated RTKs upon ligand stimulation. Rin1 plays a key role in receptor internalization and in signal transduction via activation of Rab5 and association with active form of Ras. This study has two main objectives: (1) It determines the role of Rin1 in the regulation of several RTKs focusing on insulin receptor. This was accomplished by studying the Rin1-insulin receptor interaction using a variety of biochemical and morphological assays. This study shows a novel interaction between the insulin receptor and Rin1 through the Vps9 domain. Two more RTKs (epidermal growth factor receptor and nerve growth

factor receptor) also interacted with the SH2 domain of Rin1. The effect of the Rin1-RTK interaction on the activation of both Rab5 and Ras was also studied during receptor internalization and intracellular signaling. Finally, the role of Rin1 was examined in two differentiation processes (adipogenesis and neurogenesis). Rin1 showed a strong inhibitory effect on 3T3-L1 preadipocyte differentiation but it seems to show a modest effect in PC12 neurite outgrowth. These data indicate a selective function and specific interaction of Rin1 toward RTKs. (2) It examines the role of the small molecule Dehydroleucodine (DhL) on several key signaling molecules during adipogenesis. This was accomplished by studying the differentiation of 3T3-L1 preadipocytes exposed to different concentrations of DhL in different days of the adipocyte formation process. The results indicate that DhL selectively blocked adipocyte formation, as well as the expression of PPAR γ , and C/EBP α . However, DhL treatment did not affect Rin1 or Rab5 expression and their activities.

Taken together, the data indicate a potential molecular mechanism by which proteins or small molecules regulate selective and specific RTK intracellular membrane trafficking and signaling during cell growth and differentiation in normal and pathological conditions.

TABLE OF CONTENTS

CHAPTER	PAGE
I. INTRODUCTION	1
Receptor Mediated Endocytosis	1
Epidermal Growth Factor Receptor.....	3
The Insulin Receptor.....	5
The Nerve Growth Factor Receptor.....	7
Signal Transduction and Endocytosis	8
Hyperproliferative Diseases and Cancer.....	10
Obesity and Diabetes.....	13
Brain Function and Neurogenesis.....	15
Natural products: Phytochemicals.....	17
Hypothesis and Specific Aims.....	21
II. MECHANISM OF INTERACTION BETWEEN RIN1 AND RTKS AND ITS EFFECTS TOWARD THE ACTIVITY OF RAB5 AND RAS.....	39
III. THE ROLE OF RIN1 (AND RIN1-LIKE MOLECULES) IN SIGNALING TRANSDUCTION PATHWAYS INVOLVED IN CELL DIFFERENTIATION.	66
IV. EXAMINING THE EFFECT OF DEHYDROLEUCODINE IN DIFFERENTIATION OF ADIPOSE CELLS.	80
V. DISCUSSION.....	99
FUTURE WORK.....	106
MATERIALS AND METHODS.....	107
Cloning.....	107
Construction of recombinant retroviruses and stable cell lines expressing protein constructs.....	107
Rin1 depletion in 3T3-L1 cells.....	107
Lysate preparation, SDS-PAGE and Western blotting.	108
GST-fused protein purification.	108
Pull-down assay.....	108
Rab5 activation assay.....	109
Ras activation assay.	109
Insulin receptor tail phosphorylation.....	110
Immunoprecipitation assay.	110
Yeast Two-Hybrid assay.	110
Tyrosine kinase receptor-depending signaling.....	111
3T3-L1 preadipocyte differentiation.....	111
High-performance liquid chromatography analysis.	112

Gas chromatography (GC)/flame ionization detector (FID) and GC/ mass spectrometry (MS) analysis of DhL derivatives.	113
DhL derivatives: compound identification.....	113
Effect of DhL in Rin and Rab5 expression and activation.	114
Adipogenesis quantification.	114
Neurogenesis.....	115
Statistical analysis.....	115
REFERENCES	116
APPENDICES	133
VITA	134

LIST OF FIGURES

FIGURE	PAGE
Figure 1.1. Receptor mediated endocytosis.	24
Figure 1.2. EGFR structure.....	25
Figure 1.3. EGFR signaling.	26
Figure 1.4. Insulin receptor structure.	27
Figure 1.5. Insulin receptor signaling.....	28
Figure 1.6. TrkA structure.....	29
Figure 1.7. TrkA signaling.....	30
Figure 1.8. Ras signaling.....	31
Figure 1.9. Rab5/ Ras cycle.	32
Figure 1.10. Rab5 GAPs proteins.....	33
Figure 1.11. Rab5 GEFs proteins.....	34
Figure 1.12. Rin1 domains.	35
Figure 1.13. Rin protein family.....	36
Figure 1.14. Adipogenesis signaling.....	37
Figure 1.15. Transcription factors involved in adipogenesis.	38
Figure 2.1. Immunoprecipitation assay between Rin1 (WT and domains) and the EGF receptor.	47
Figure 2.2. GST-pulldown assay between EGF receptor and Rin1 mutants.	48
Figure 2.3. Immunoprecipitation assay between Rin1 (WT and domains) and the insulin receptor.	49
Figure 2.4. Yeast two hybrid system using Rin1 constructs and the insulin receptor cytoplasmic tail.	50

Figure 2.5. GST-pulldown assay between the insulin receptor and Rin1 mutants.....	51
Figure 2.6. GST-pulldown assay between the NGF receptor and Rin1 mutants.....	52
Figure 2.7. Rab5 activation in NR6 cells overexpressing EGFR and Rin proteins.....	53
Figure 2.8. Rab5 activation in NIH3T3 cells overexpressing Rin1.....	54
Figure 2.9. Ras activation in NR6 cells overexpressing Rin1.....	55
Figure 2.10. EGFR signaling in NR6 cells overexpressing Rin1.....	56
Figure 2.11. EGFR signaling in NR6 cells overexpressing Rin2.....	57
Figure 2.12. EGFR signaling in NR6 cells overexpressing Rin3.....	58
Figure 2.13. IR signaling in NIH3T3 cells overexpressing Rin1.....	59
Figure 2.14. IR signaling in NIH3T3 cells overexpressing Rin2.....	60
Figure 2.15. IR signaling in NIH3T3 cells overexpressing Rin3.....	61
Figure 2.16. IR signaling in NIH3T3 cells overexpressing Rin1:R94A mutant....	62
Figure 2.17. IR signaling in NIH3T3 cells overexpressing Rin1: Y561F mutant.	63
Figure 2.18. IR signaling in NIH3T3 cells overexpressing Rin1: T580A mutant.	64
Figure 2.19. IR signaling in NIH3T3 cells overexpressing Rin1:R629A mutant..	65
Figure 3.1. Rab5/ Rin1 endogenous expression during 3T3-L1 differentiation...	71
Figure 3.2. Lipid droplet quantification in cells overexpressing Rin proteins and Rin1 domains.....	72
Figure 3.3. Different constructs of the domains of Rin1.....	73
Figure 3.4. Adipogenic markers expression in cells overexpressing Rin1.....	74
Figure 3.5. Adipogenic markers expression in cells overexpressing the different Rin1 domains.....	75
Figure 3.6. Lipid droplet quantification in cells overexpressing Rin1 mutants.....	76

Figure 3.7. Adipogenic markers expression in cells overexpressing the different Rin1 mutants.	77
Figure 3.8. CREB activation in cells overexpressing Rin1 and control cells.	78
Figure 3.9. Effect of Rin proteins in PC12 differentiation.	79
Figure 4.1. Dehydroleucodine inhibited adipogenesis of 3T3-L1 preadipocytes without reducing cell viability.	89
Figure 4.2. Dehydroleucodine blocked the formation of lipid droplet by induction media in 3T3-L1 cells.	90
Figure 4.3. Dehydroleucodine attenuated the expression of PPAR γ during 3T3-L1 preadipocyte differentiation.	91
Figure 4.4. Dehydroleucodine selectively alters the expression of PPAR γ , C-EBP α and AMPK α	92
Figure 4.5. Dehydroleucodine blocked adipocyte differentiation in a time-dependent manner.	93
Figure 4.6. Dehydroleucodine alters the expression of FAS.	94
Figure 4.7. GC analysis of dehydroleucodine and 11,13-dihydrodehydroleucodine epimers.	95
Figure 4.8. 11,13-Dihydro-dehydroleucodine inhibited 3T3-L1 preadipocyte differentiation.	96
Figure 4.9. Effect of dehydroleucodine in Rab5 expression and activation.	97
Figure 4.10. Effect of dehydroleucodine in Rin1 expression.	98

ABBREVIATIONS

ALS:	Amyotrophic lateral sclerosis
AP2:	Adaptor protein 2
BAT:	Brown adipose tissue
BDNF:	Brain-derived neurothropic factor
BSA:	Bovine serum albumin
BCL-2:	B-cell lymphoma 2
C/EBP α :	CCAAT/ enhancer- binding protein
Ca ⁺² :	Calcium
CREB:	cAMP response element-binding protein
DhL:	Dehydroleucodine
EEA1:	Early endosome antigen 1
EGF:	Epidermal growth factor
EGFR:	Epidermal growth factor receptor
Erk:	Extracellular signal regulated kinase
FGF:	Fibroblast growth factor
FGFR:	Fibroblast growth factor receptor
FL:	Full length
GAP:	GTPase activating protein
GDP:	Guanosine di phosphate
GEF:	Guanine nucleotide exchange factor
GPCR:	G-protein coupled receptor
Grb2:	Growth factor receptor bound protein 2

GSK3: Glycogen synthase kinase 3

GTP: Guanosine tri phosphate

IAPs: Inhibitor of apoptosis proteins

IGF: Insulin-like growth factor

IGFR: Insulin-like growth factor receptor

IR: Insulin receptor

IRS: Insulin receptor substrate

JNK: c-Jun kinase

MACS: Macrocephaly, alopecia, cutis laxa and scoliosis

MAPK: Mitogen activated protein kinase

NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells

NGF: Nerve growth factor

NGFR: Nerve growth factor receptor

PBS: Phosphate buffered saline

PC12: Rat adrenal pheochromocytoma 12

PLC: Phospholipase C

PDGF: Platelet-derived growth factor

PDGFR: Platelet-derived growth factor receptor

PI3K: Phosphoinositide 3 Kinase

PKC: Protein kinase C

PPAR γ : Peroxisome proliferator-activated receptor

RA: Ras association domain

Rabex-5: Rabaptin-5 associated exchange factor for Rab5

Rap6: Rab5 activating protein 6

Ras: Rat sarcoma

Rin: Ras interference

Rin1: Ras interference 1

RTK: Receptor-tyrosine kinases

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SH2: SRC homology 2

Src: Sarcoma

STAM: Signal-transducing adaptor molecule

TBC: Tre-2/Bub2/Cdc16 domain

TGF: Transforming growth factor

TGF α : Transforming Growth Factor alpha

Trk: Tropomyosin-receptor-kinase

UCP-1: Uncoupling protein-1

VPS9: Vacuolar protein sorting 9

WAT: White adipose tissue

WT: Wild type

I. INTRODUCTION

Endocytosis is the process by which cells internalize molecules from the extracellular environment (Besterman and Low, 1983). There are two different types depending on the size of the internalized particles. Phagocytosis refers to the invagination of particles larger than 250 nanometers, while pinocytosis refers to particles less than 100 nanometers (Besterman and Low, 1983). The second category can be further divided into macropinocytosis and receptor-mediated endocytosis. The former requires actin cytoskeleton reorganization and provides a way to internalize, non-selectively, large quantities of solute molecules and membrane (Lim and Gleeson, 2011). Once inside the cell, these molecules are delivered into the lysosome, a compartment in which digestive enzymes break them down into metabolites that are released into the cytosol as they are needed for cell metabolism. Insulin (Maxfield et al., 1978) and growth factors such as brain-derived neurotrophic factor (BDNF) (Patapoutian and Reichardt, 2001), fibroblast growth factor (FGF) (Ornitz and Itoh, 2001), platelet-derived growth factor (PDGF) (Hannink and Donoghue, 1989), insulin-like growth factor (IGF) (Hoppener et al., 1985), epidermal growth factor (EGF) (Harris et al., 2003), and nerve growth factor (NGF) (Allewa and Santucci, 2001) are examples of molecules that use this process for their internalization.

Receptor Mediated Endocytosis

Receptor-mediated endocytosis is initiated when a ligand binds to its receptor (Figure 1.1), which in turn initiates a series of protein-protein

interactions responsible for numerous cellular processes including cell proliferation, differentiation, growth, homeostasis, and apoptosis through the regulation of target genes. Receptor mediated endocytosis can be a constitutive or an activated process as in the case of transferrin (Goldstein et al., 1985) and EGF (Barbieri et al., 2003; Dunn and Hubbard, 1984; Schlessinger, 1981), respectively.

Receptor-tyrosine kinases (RTKs) are internalized by receptor-mediated endocytosis. They are membrane-bound receptors characterized by their intrinsic kinase activity. They share a common structure characterized by 1) an extracellular region that interacts directly with the ligand; 2) a hydrophobic region that transverses the lipid bilayer; and 3) a cytoplasmic region that interacts with cytosolic molecules. Activated RTKs are responsible for multiple cellular processes including cell growth and apoptosis (Carpenter and Cantley, 1996; Riese and Stern, 1998; Zhou et al., 2000). Examples of this type of receptor are fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), insulin-like growth factor receptor (IGFR), the epidermal growth factor receptor (EGFR), insulin receptor (IR), and the nerve growth factor receptor (NGFR). In general, the ligand binds to the extracellular domain of the receptor, triggering receptor dimerization, which in turn activates specific catalytic sites on the cytoplasmic face of the receptor, resulting in autophosphorylation of the receptor's cytoplasmic tail and activation of multiple signal transduction cascades through protein-protein interactions (Hubbard and Miller, 2007).

Epidermal Growth Factor Receptor

The EGFR belongs to a family composed of four types of receptors: ERBB1 (EGFR), ERBB2, ERBB3, and ERBB4. From these, ERBB2 does not bind any known EGF-like ligand; ERBB3 is kinase inactive, and ERBB4 is the only one autonomous (Bubliil and Yarden, 2007). Upon ligand stimulation, ERBB4 is the only one that does not have to form a dimer and that ERBB2 and ERBB3 homodimers are not feasible because they lack ligand binding and kinase activity, respectively. As mentioned before, EGFR has the three different domains characteristic of RTKs: a ligand-binding extracellular domain, a hydrophobic transmembrane domain, and the tyrosine kinase activity cytoplasmic domain (Figure 1.2) (Carpenter, 1987; Downward et al., 1984; Hunter and Cooper, 1985).

The EGFR family recognizes different types of ligands including EGF, Transforming Growth Factor-alpha (TGF-alpha), amphiregulin, betacellulin, epiregulin, and epigen (Nair, 2005). These receptors have a diffuse distribution on the cell surface when the ligand is not present. Upon EGF stimulation, the receptors cluster (homo or heterodimerization) in clathrin-coated pits to be internalized (Heath et al., 2003). Even though EGFR signaling is downregulated by the receptor internalization and targeting to the endosomal compartment (Holbro and Hynes, 2004), it is now known that it continues generating signals until its degradation in the lysosome (Avraham and Yarden, 2011). At physiological levels of EGF (low doses 1-2 ng/ml), the receptor is internalized mainly through clathrin-coated pits (Sigismund et al., 2008). The process of

internalization is triggered by the direct interaction of the receptor tail with the adaptor protein AP2, which recruits clathrin to the plasma membrane creating the coated pit. The GTPase Dynamin is the one responsible for the vesicle fission from the plasma membrane forming the clathrin-coated vesicle (Schmid, 1997). Once inside the cell, the coat disassemble takes place, and the vesicle fuses with the early endosome continuing with the internalization process and the signaling cascade activated by the ligand-receptor complex (Kaksonen et al., 2006).

Epidermal growth factor receptor signaling activates different types of transcription factors including c-jun, c-fos, c-myc, and NF- κ B, (Holbro and Hynes, 2004). Transcription factor regulation is done by the activation of key signaling pathways such as Erk and Akt, among others (Avraham and Yarden, 2011). Epidermal growth factor receptor signaling plays a key regulatory role in biological processes such as migration, proliferation, and resistance to apoptosis (Wieduwilt and Moasser, 2008). It is also a prominent regulator of cell lineage determination in different types of tissue (Figure 1.3). Overexpression of EGFR has been found in different types of cancer, specifically in breast and ovarian tumors (Nair, 2005). Recent studies have demonstrated the crosstalk between EGFR and GPCR (G-protein coupled receptors) and its importance in several cancers such as astrogloma cells and ovarian cancer cells. The crosstalk between EGFR and GPCR is the result of EGFR activation by GPCR agonists (Gschwind et al., 2001). Aberrant EGFR activation through non-EGF may inactivate phosphatases that control the receptor intrinsic kinase activity, shifting the autophosphorylation/dephosphorylation equilibrium toward the active state

(Fischer et al., 2003). Different key cytoplasmic proteins, such as Src and PKC, are involved depending on the system and the signaling context (Gschwind et al., 2001). In summary, GPCR-induced activation of EGFR and downstream signaling through Src and PKC regulation are found in more than 60 human carcinoma cell lines derived from different tissues (Fischer et al., 2003).

The Insulin Receptor

The insulin receptor exists as a dimer composed of two α (extracellular) and two β (intracellular) subunits linked by a disulfide bond (Figure 1.4). There are two isoforms of the receptor that differ in 12 amino acids in the carboxyl terminus of the extracellular domain (the α -subunit). Some studies suggest that one isoform may signal more efficiently to metabolic processes while the other to mitogenic events (Belfiore et al., 2009). Despite the fact that both isoforms can form hetero- or homodimers to initiate the signaling cascade, they have higher affinity for only one ligand molecule upon stimulation (Siddle, 2011). As in the case of EGFR, the insulin receptor gets internalized through clathrin-coated pits and clathrin-coated vesicles until its fusion with early endosome, late endosome and finally the lysosome where its degradation takes place (Ceresa et al., 1998). Once the receptor gets activated upon insulin stimulation, it will initiate the signaling cascade as with other types of RTKs, with the exception of the insulin receptor substrate (IRS) protein recruitment. The activated receptor will recruit and phosphorylate IRS proteins, which are responsible for amplifying the signal by the recruitment and activation of SH2 domain-containing proteins (Su et al., 2006). Ultimately these proteins will regulate the activity of several transcription

factors including TORC1 and FOXO through the PI3K/ Akt and GRB2-SOS/Ras cascades (Figure 1.5) (Cheng et al., 2010).

Insulin signaling plays a major role in several biological processes including protein synthesis, lipid metabolism, glycolysis and glucose storage in muscle, liver and adipose tissue (Shaham et al., 2008). In muscle cells, insulin activates GLUT4 transporters from the cytoplasm to the plasma membrane increasing the intracellular concentration of glucose (Summers et al., 1998). In the liver, insulin stimulates glycogen synthesis by promoting the phosphorylation of glucose, confining it within the cell (Hua, 2010). In adipose tissue, insulin promotes the storage of lipids in the form of triglycerides by phosphorylating CREB protein and activating key adipogenic transcription factors such as PPAR γ and C/EBP α as explained below. Malfunction of these processes is associated with systemic disorders such as hypertension, obesity, diabetes, cardiovascular disease, infertility and neurodegeneration (Cheng et al., 2010; Reaven and Tsao, 2003; Stumvoll et al., 2005). Recent studies demonstrated a crosstalk between insulin-like growth factor receptor (IGFR) and EGFR and its role in some types of hyper-proliferative diseases such as pancreatic and breast cancer (Ueda et al., 2006). Insulin-like growth factor receptor, as well as the insulin receptor, is a member of the insulin-like growth factor receptor family. Both receptors have not only structural similarities, but also activate similar signaling cascades. It is believed the biological difference between the two receptors lies in its different tissue distribution and substrate recruitment (Werner et al., 2008). Depending on

the tissue and signaling context, it would not be unexpected to see a crosstalk not only between EGFR and IGFR but also with the insulin receptor.

The Nerve Growth Factor Receptor

The nerve growth factor receptor family is composed of the tropomyosin receptor kinase (Trk) and the p75 protein receptor (Sofroniew et al., 2001). There are three different Trk receptors, each composed of a single trans-membrane region and a cytosolic kinase domain (Figure 1.6). Tropomyosin receptor kinase A (TrkA) gets activated primarily by NGF, TrkB by BDNF, and neurotrophin 4/5 and TrkC by neurotrophin 3. The Trk family of tyrosine kinase receptors regulates the development and maintenance of the peripheral and central nervous system (Brodeur et al., 2009). This study will focus on TrkA. Tropomyosin receptor kinase A also uses the clathrin-mediated pathway for its internalization in the same way as EGFR and the insulin receptor (Grimes et al., 1996). The primary roles of NGF/TrkA signaling are to promote cell survival, neurite outgrowth, neuronal differentiation, and activity-dependent plasticity of sympathetic neurons (Brodeur et al., 2009). These biological processes are obtained by the activation of three major signaling pathways: PI3K/Akt, Erk, and Phospholipase C (PLC)/protein kinase C (PKC), respectively (Figure 1.7). Malfunction of these signaling pathways is related to various neurodegenerative diseases such as ALS (amyotrophic lateral sclerosis), Parkinson's, Huntington's and Alzheimer's diseases as well as in different types of cancer including thyroid carcinoma and neuroblastoma (Brodeur et al., 2009; Connor and Dragunow, 1998; Kruttgen et al., 2006; Siegel and Chauhan, 2000).

Signal Transduction and Endocytosis

Different proteins are involved in the signaling cascades activated by RTKs. After receptor activation, multiple proteins are recruited and play key roles in the activated signal cascade (Perona, 2006). Studies have shown the important role the small GTPases Rab and Ras proteins play during receptor-mediated endocytosis, membrane trafficking, vesicle transport, and signal transduction (Figure 1.8) (Agola et al., 2011; Hancock, 2003). These proteins cycle between their active form, bound to GTP, and their inactive form, bound to GDP. Guanine nucleotide exchange factors (GEFs) promote the exchange from GDP to GTP, activating these small GTPases. On the other hand, GTPase activating proteins (GAPs) promote the hydrolyzation of GTP, inactivating them (Figure 1.9).

Studies have shown the important regulatory role of Rab5 in the endocytic rate and early endosome fusion (Bucci et al., 1992; Li et al., 1994; Li and Stahl, 1993; Roberts et al., 1999). As with other GTPases, Rab5 has a very low intrinsic GTPase activity, and depends on other enzymes such as GAPs to hydrolyze GTP, downregulating its activity. These enzymes share a common Tre-2/Bub2/Cdc16 (TBC) catalytic domain responsible for GTP hydrolyzation. Examples of Rab5 GAP proteins are RabGap5, RN-tre (Pfeffer, 2005), and TBC-2 (Chotard et al., 2010), among others (Figure 1.10). Multiple Rab5 GEFs have been identified. These proteins are characterized by sharing a highly conserved Vps9 (vacuolar protein sorting 9) domain that catalyzes the exchange from GDP to GTP, activating Rab5. Examples are the yeast homolog Vps9p (Hama et al.,

1999), Rabex-5 (Carney et al., 2006), Rin1 (Han and Colicelli, 1995; Tall et al., 2001), Alsln2 (Otomo et al., 2008), RME-6 (Sato et al., 2005), and Rap 6 (Hunker et al., 2006a; Sato et al., 2005) (Figure 1.11).

Several of these proteins contain, besides the Vps9, other domains that interact and sequester other signaling molecules. Thus, their localization on early endosomes allows them to increase endocytosis by the activation of Rab5, and to regulate specific signaling cascades. The same is the case of Ras Interference 1 (Rin1), which consists of four domains. First, the SH2 domain (SRC Homology 2) located at the amino-terminus mediates a direct interaction with RTKs such as the IR and EGFR (Barbieri et al., 2003). Second, the Proline Rich (PR) interacts with proteins such as the signal-transducing adaptor molecule (STAM) (Kong et al., 2007). Third, the Vps9 domain provides the site for the interaction and subsequent activation of Rab5, as explained above. Finally, the carboxy-terminus houses the Ras-Association (RA) domain, which interacts with the active form of Ras (Barbieri et al., 2003) (Figure 1.12). Ras Interference 1 competes with the Ras downstream effector Raf, inhibiting the its signaling cascade (Han and Colicelli, 1995). Thus, Rin1 is involved in both processes by its direct interaction through its three different domains with Rab5 (endocytosis) and Ras (cell signaling). Furthermore, it has been shown that several important residues of Rin1 play a key role in the activation of Rab5 (Galvis et al., 2009), and that it is potentiated by the interaction of Rin1 with the active form of Ras (Tall et al., 2001).

Ras Interference 1 is part of a protein family composed of two other members: Rin2 and Rin3 (Figure 1.13). The former is also a Rab5-binding protein, but unlike Rin1, it has more affinity to Rab5-GTP; however, it also enhances the guanine nucleotide exchange activity of Rab5. Deficiency of Rin2 has been related to a disorder of elastic tissue known as macrocheily, alopecia, cutis laxa and scoliosis syndrome (MACS) (Basel-Vanagaite et al., 2009). Ras Interference 2 has the same domain structure as Rin1, with an extra proline rich domain. The same structure is observed in Rin3, except that it is composed of three proline rich domains. It also binds the active form of Rab5 rather than the inactive one, just like Rin2 (Kajiho et al., 2003). It seems that Rin3 has a specific expression pattern restricted to mast cells, controlling basic functions as well as malfunction such as chronic inflammation and cell hyper proliferation (Janson et al., 2012).

Hyperproliferative Diseases and Cancer

Hyperproliferative diseases such as cancer are characterized by unregulated cell growth (Sebastian et al., 2006). Alterations in the equilibrium between cell proliferation and programmed cell death results in tumor formation as a consequence of uncontrolled cell division. Development and growth of cancer cells is produced by genetic modifications in signaling pathways that regulate cell proliferation, differentiation, survival and motility (Nicholson et al., 2001). In most cases these signaling pathways are activated by RTKs, and their role in cancer development is, at least in part, a result of the receptor abnormal expression and activation. Aberrant EGFR (and its downstream signaling)

activation has been found in numerous types of cancer (Nair, 2005). The malfunction of EGFR has been supported by epidemiological evidence in human tumors, which demonstrated the contribution of abnormal EGFR expression and signaling in numerous epithelial malignancies including breast cancer (Suo and Nesland, 2002), squamous skin carcinomas (Merlino et al., 1985), renal carcinoma (Moch et al., 1997), head and neck tumors (Rogers et al., 2005), among others (Sebastian et al., 2006). The anomalous EGFR functioning includes activating mutations and receptor overexpression, which contributes to oncogenesis by inducing cell proliferation and apoptosis resistance (Henson and Gibson, 2006).

Overactivated receptors such as EGFR and IGFR transform a normal cell to a malignant one by providing sustained signals that promote cell proliferation, anti-apoptosis, angiogenesis and metastasis, which are the basics properties of cancer development and progression (Arteaga, 2002). The GTPase Ras is a key regulator of signal transduction within the cell. It is a small GTPase that modulates signal activation upon membrane receptor stimulation. It has three isoforms: H-Ras, K-Ras, and N-Ras, all of which are involved in different types of tumors when over activated (Colicelli, 2004). The main deregulated signaling pathways involved in oncogenesis are the Raf-Erk, PI3K-Akt, and Jak-Stat (Nair, 2005). The first one promotes cell proliferation and inhibits programmed cell death, by increasing the transcription of the BCL-2 family members and IAPs (inhibitor of apoptosis proteins) resulting in cell survival and proliferation (Henson and Gibson, 2006). The PI3K-Akt pathway also promotes cell survival and cell

growth through the activation of anti-apoptotic genes such as NFkB and CREB. Akt also phosphorylates the GSK3 kinase, inhibiting it, blocking transcriptional activity and metabolism regulation (Henson and Gibson, 2006). Finally, the Jak-Stat pathway promotes cell survival by the activation of survival genes through the translocation of Stat to the nucleus (Henson and Gibson, 2006).

Malfunction of Rab5 has been related to different types of cancer. Upregulation of Rab5 expression has been associated with thyroid autonomous adenomas (Stein et al., 2003), and Rab5 activation has been shown to promote focal adhesion disassembly, enhancing migration and invasion of cancer cells (Mendoza et al., 2013). Furthermore, Rin1 has also been related to multiple types of cancer. Rin1 localization has been shown to play an important role in colon cancer cells carcinogenesis (Inoue et al., 2011), while in breast cancer it seems to function as a tumor suppressor by negatively regulating tumor cell invasive growth (Milstein et al., 2007). On the other hand, on some non-small lung adenocarcinomas cell lines, upregulation of Rin1 expression seems to increase its proliferative properties (Tomshine et al., 2009). Taking these data together, it seems that the role of Rin1 in cancer progression is tissue-dependent and is related to the role of Rin1 as a Rab5 activator and a Ras effector.

Therefore, studying of the signaling pathways and its relationship with Rin1 is important to understand the molecular mechanisms of hyper proliferative diseases such as cancer.

Obesity and Diabetes

Obesity is a medical condition characterized by the excess accumulation of body fat. Fat deposits can be subcutaneous (beneath the skin) or visceral (around internal organs). The former is related to many of the obese-related pathologies such as cancer, heart disease and diabetes, among others (Hassan et al., 2012). Diabetes is a metabolic disorder characterized by high blood glucose levels as a consequence of the inability of cells to absorb glucose. The high glucose levels happen because, at least in part, pancreatic beta cells are not able to produce insulin, or because cells cannot respond to the one produced properly, also known as insulin resistance (Guillausseau et al., 2008).

Adipogenesis is the formation of adipocytes (fat cells) from preadipocytes (Rosen and Spiegelman, 2000). Different types of hormones and signaling cascades, including insulin, regulate this process (Figure 1.14). There are two main types of adipose tissue: white fat (WAT) and brown (BAT) tissue. White fat tissue stores excess energy in the form of triglycerides in large lipid droplets. White adipose tissue storage is important because by storing lipid excess, it prevents lipid accumulation in other tissues (Koppen and Kalkhoven, 2010). In addition, lipases hydrolyze triglycerides of WAT tissue into fatty acids that can be oxidized to generate energy as needed (Koppen and Kalkhoven, 2010; Rosen and Spiegelman, 2006). Recent studies have shown that WAT is not only the primary lipid storage, but that it also functions as an endocrine organ releasing different types of adipokines that regulate immune responses, blood pressure, angiogenesis, bone mass, thyroid and reproductive functions (Trayhurn, 2005).

On the other hand, BAT tissue is specialized in defense against cold and works as a protection against lipid accumulation by dissipating energy (instead of storing it) in the form of heat (Rosen and Spiegelman, 2000). This is achieved by an increase in the number of mitochondria present in this type of cell. Specifically, high expression of uncoupling protein (UCP)-1 dissipates the proton gradient across the inner mitochondrial membrane during the electron movement in the respiratory chain (Garlid et al., 1998). In BAT tissue, triglycerides are stored in multiple small lipid droplets (Cannon and Nedergaard, 2004). These two types of adipose tissue are found in multiple locations within the body. Usually they are found in areas rich in loose connective tissue (such as between muscle and dermis) and around major organs such as the heart and kidneys (Rosen and Spiegelman, 2000). White adipose tissue location has been associated with different types of diseases. Visceral adipose tissue is associated with an increase risk of cardiovascular malfunctions and insulin resistance, while subcutaneous tissue accumulation is not (Rosen and MacDougald, 2006).

Adipocyte differentiation occurs in two separate phases, each one marked and controlled by different transcription factors. The first phase is characterized by cell growth arrest followed by one or two rounds of mitosis known as clonal expansion. The second phase starts by a second round of growth arrest and is followed by the biochemical and morphological changes necessary to give rise to the adipocyte phenotype (Rosen and Spiegelman, 2000). The first factors to be activated during adipogenesis are the CCAAT/enhancer-binding proteins (C/EBP) β and γ (Evans et al., 2004). The early and transient activation of

C/EBP β and γ is thought to be responsible for the clonal expansion, characteristic of the first phase, and for the induction of PPAR γ and C/EBP α expression (Lane et al., 1999). Peroxisome proliferator-activated receptor γ is activated first and induces the expression of C/EBP α that at the same time, in a positive control, helps to maintain a high PPAR γ expression level. These two transcription factors mediate the expression of adipocyte-specific genes that lead to the adipogenic differentiation phenotype (Hwang et al., 1997). The function of Erk activity is not clear yet. Some studies suggest a key role in the clonal expansion in the first phase of differentiation. Then it must be downregulated because Erk activity leads to phosphorylation of PPAR γ , inhibiting adipocyte differentiation (Rosen and MacDougald, 2006) (Figure 1.15).

Malfunction in the equilibrium of adipose tissue can lead to numerous diseases including obesity, diabetes, insulin resistance, glucose intolerance, hypertension, nonalcoholic fatty liver and cardiovascular disease (Wang, 2010). Hence, it is necessary to study this biological process in detail as well as the proteins involved.

Brain Function and Neurogenesis

Neurogenesis is the process by which new neurons are formed in the nervous system (Gotz and Huttner, 2005). Long branches that develop from the cell body characterize neuron cells. During neurogenesis, intracellular organization and assembly of microtubules and filamentous proteins of the cytoskeleton such as Maps (microtubule associated proteins) are involved in neurite growth (Biocca et al., 1983). Nerve growth factor is an essential

neurotrophin for cell differentiation and survival (Liu et al., 2007), it activates numerous signaling pathways by its binding to TrkA as explained above. Studies in PC (rat adrenal pheochromocytoma) 12 cells showed that the internalization of the TrkA/NGF complex and formation of the signaling endosome is essential for NGF-induced differentiation (Grimes et al., 1996). These signaling endosomes are involved in long distance retrograde transport in axons, compared with the short distance traveled by early endosomes in regular cells. Interestingly enough, a population of this neuronal signaling endosomes is also Rab5-positive (Liu et al., 2007). This suggests that Rab5 activity must be attenuated to prevent the transition to late endosomes and further degradation of the receptor complex terminating the signal. Low levels of Rab5-GTP found in differentiated PC12 cells suggest the NGF-induced signaling downregulation of Rab5 activity (Liu et al., 2007).

The ligand/receptor complex recruits proteins that activate the Ras and Rap1 GTPases, the ones that in turn activate Raf, PI3K, and PLC among other signaling proteins. These are the main pathways responsible for the transcription of specific genes that commit to the differentiation process (Faigle and Song, 2013; Wang et al., 2006).

Neurodegenerative diseases such as Parkinson's (Goedert, 2001), Alzheimer's (Hardy and Selkoe, 2002), and Amyotrophic Lateral Sclerosis (ALS) (Qiang et al., 2013) are characterized by a loss in the neuronal population within the nervous system. The cell loss is responsible for the cognitive and motor function damage that comes as symptoms of such diseases (Abdipranoto et al.,

2008). Studies have shown neurogenesis in the adult nervous system (Eriksson et al., 1998), demonstrating the ability of some neuronal populations in the adult brain to regenerate themselves. These findings may suggest another approach to understand and cure neurodegenerative diseases: instead of focusing on slowing cell loss, the control and cure of these diseases can be in enhancing neurogenesis to replenish the neuronal population lost.

Natural products: Phytochemicals

The importance of natural products in medicine as well as in the agroindustry has led to numerous studies on the synthesis and biological activities of these substances. Yet little is now known about their actual roles in nature, particularly their use as alternative medicine.

Many natural products show anti-obesity (Vasudeva et al., 2012) and anti-tumoral activities of varying mechanisms (Aravindaram and Yang, 2010). Possibly a method to find a more efficient way to treat diseases like obesity and cancer, and more important, to achieve the synergistic effects of natural products should be to search for treatments using multiple products and/ or products having multiple biological activities.

Sesquiterpene lactones are secondary metabolites found in most species of Compositae (*Asteraceae*), and there are more than 6,000 sesquiterpene lactones with known structures. Parthenolide was first reported as a new sesquiterpene lactone from Feverfew (*Tanacetum parthenium*) (Pareek et al., 2011). Its organization revealed a structure of an alpha-methylene-gamma-lactone. One of the most important characteristics of sesquiterpene lactones is

their anti-tumor activity (Zhang et al., 2005). Interestingly, a number of naturally occurring sesquiterpene lactones with the alpha-methylene-gamma-lactone (alpha-methylenenbutyrolactone) structure, such as parthenolide and helenalin, were reported to have potent cytotoxic activity toward several tumor cell lines as well as human cells transformed with Simian Virus (Lee et al., 1971; Wu et al., 2006). Several studies have proposed that the anti-tumor activity of sesquiterpene lactones might occur at the DNA replication as well as other intracellular targets (Ross et al., 1999; Wiedhopf et al., 1973; Woynarowski and Konopa, 1981; Zhang et al., 2004).

A sizeable body of written work indicates that exponential progress has been made concerning the knowledge of bioactive components in plant foods and their relation to obesity (Rayalam et al., 2008). Because adipogenesis is directly related to adipocyte differentiation and maturation, the induction of apoptosis as well as the inhibition of adipogenesis at various stages of the adipocyte life cycle may be target pathways for treating obesity.

As described above, in cancer cells, phytochemicals have a propensity to increase the curative effect by either blocking one or more molecular targets of the signal transduction pathway of high proliferative cancer cells. However, dietary natural products have shown interesting effects on adipose tissue such as inducing apoptosis and decreasing lipid content (Nelson-Dooley et al., 2005), affecting a number of complex interconnected cell signaling pathways involved in regulating adipogenesis. Thus, natural compounds may act at single or multiple

target sites in the adipocyte or cancer cells life cycle associated with key physiological processes.

About 25 percent of commonly used drugs to treat diseases such as diabetes, obesity and cancer come from plant extracts (Balandrin et al., 1985). Treatments to fight diseases such as obesity, and others illness related to it, focus on either to inhibit the formation or to promote apoptosis of adipose tissue. Plant extracts work on these two aspects with different mechanisms: to prevent the disease or to treat it. Some plants extracts containing caffeine, are metabolic stimulants by inhibiting the degradation of intracellular cAMP that causes an increase in energy expenditure and decreases energy intake by reducing food intake (Dulloo, 1993; Racotta et al., 1994). Extract of *Hoodia gordonii* works as an appetite suppressant in rats, just as caffeine, by increasing ATP concentration in hypothalamic neurons that regulate food intake (MacLean and Luo, 2004).

Studies showed the role of *Siraitia grosvenori* and *Stachytarpheta cayennensis*, among others, in insulin sensitivity and blood glucose control (Adebajo et al., 2007; Suzuki et al., 2007), even though the mechanism of regulation is not yet understood. Plants such as *Cissus quadrangularis*, *Aralia mandshurica*, *Kochujang* and *psyllium* showed a reduction in triglycerides and cholesterol levels in serum (Abidov et al., 2006; Ahn et al., 2006; Moreno et al., 2003; Oben et al., 2007).

Regulation of these signaling pathways, which can modulate adipogenesis at different developmental stages, is essential for several cellular processes. Several plant extracts have an effect on the first phase of differentiation.

Flavonoids (Hsu and Yen, 2006) and CLAs (Hsu and Yen, 2006) have been shown to inhibit 3T3-L1 preadipocyte proliferation and to induce apoptosis. In the presence of genestein, mitotic clonal expansion of 3T3-L1 preadipocytes is inhibited (Harmon and Harp, 2001) and apoptosis of mature preadipocytes is enhanced in the presence of esculetin (Yang et al., 2006). Extracts such as EGCG, genistein and capsaicin were shown to activate AMPK, inhibiting phase two of adipogenic differentiation (Hwang et al., 2005).

Natural products or their derivatives compose 67 percent of the anti-cancer drug treatments (Balunas and Kinghorn, 2005; Ghantous et al., 2010). Plant metabolites containing sesquiterpene lactones have been studied as possible treatments for several human diseases, including inflammation, infections, headaches and cancer (Ghantous et al., 2010). Early work showed the importance of the biological activity of the α -methylene- γ -lactone structure in sesquiterpene lactones known to be cytotoxic to tumors (Hartwell and Abbott, 1969). One of the benefits of the plant extract-based treatments in clinical trials is their ability to target tumor cells and cancer stem cells, without affecting normal healthy cells (Jordan, 2006; Kawasaki et al., 2009; Zhou and Zhang, 2008). The following are some examples of ongoing clinical trails based on plant extracts (*Artemisia annua*, *Thapsia* and *Tanacetum parthenum*, among others): laryngeal carcinomas, uveal melanomas, pituitary macroadenomas, lupus nephritis and breast, colorectal and nonsmall cell lung cancers (Berger et al., 2005; Lu, 2002; Singh and Panwar, 2006; Zhang et al., 2008). Even though the mechanism of action is still not fully understood, it seems that these plant extracts target the

sarco/ER calcium ATPase pump, proteases secreted by cancer cells, NF- κ B signaling, p53 and transferrin receptor activity (Christensen et al., 2009; Denmeade and Isaacs, 2005; Efferth, 2006; Gopal et al., 2009; Hehner et al., 1998; Nakase et al., 2009; Pajak et al., 2008).

Hypothesis and Specific Aims

As discussed above, receptor-mediated endocytosis initiates a series of protein-protein interactions responsible for essential cellular processes including cell proliferation, differentiation, and apoptosis through the regulation of selective and specific genes. Tyrosine kinase regulation changes gene modulation, which in turn affects whole-body metabolism involved in the pathogenesis of a variety of metabolic diseases, including Type 2 diabetes, cardiovascular disease, hypertension, stroke and some forms of cancer.

The actual knowledge on receptor tyrosine kinases and small GTPases interaction is limited and holds high interest among cellular and molecular biologists. Specifically, how activation of receptor tyrosine kinases leads to upregulation of small GTPases, which orchestrate and regulate several signal transduction pathways, remains elusive and far from clear. Thus, the rationale for the dissertation is that the interaction between receptor tyrosine kinases, small molecules and regulators of small GTPases (these factors orchestrate and regulate intracellular membrane traffic, via intermediate multifunctional proteins) are most likely to be essential for modulation of several cellular events in normal and diseased cells.

The first part of this study addresses whether membrane targeting of Rin1 by receptor tyrosine kinases plays a key role on the spatial and temporal regulation of Rab5 and Ras activities. Furthermore, it will examine how regulation of these small GTPases is connected to selective specific signaling pathways. Therefore, this part of the study will:

1. Determine the mechanism of Rin1-RTKs interaction and its downstream effect in receptor internalization and activation of signaling cascades.
2. Elucidate the role of Rin1 in different signaling pathways involved in cell differentiation.

The second part of the dissertation focuses on anti-differentiation properties of a natural compound Dehydroleucodine (DhL) on *in vitro* adipogenesis using 3T3-L1 preadipocytes. As people become obese, their adipocytes enlarge and cause molecular and cellular alterations such as an increase in lipid accumulation and the deregulation of several signaling pathways involved in the pathogenesis of a variety of metabolic diseases. Hence, understanding the origin and development of adipocytes may be critical to the analysis and treatment of many chronic diseases. Therefore, this part of the study aims to:

1. Determine the effect of DhL as a potent and selective inhibitor for adipogenesis.
2. Elucidate the potential(s) mechanism(s) of action of DhL on adipogenesis.

This thesis is divided into five chapters:

Chapter 1 is the introduction and literature review, which describes the importance of 1) RTK-membrane trafficking and signaling in normal and diseased cells and 2) natural products in biological processes.

Chapter 2 examines the mechanism of interaction between Rin1 and RTKs and its regulation toward the activity on Rab5 and Ras in RTK internalization and signal transduction pathways, respectively.

Chapter 3 focuses on the role of Rin1 (and Rin1-like molecules) in different signaling pathways involved in cell differentiation.

Chapter 4 examines the effect of the small molecule Dehydroleucodine in differentiation of adipose cells.

Chapter 5 summarizes the findings and significance of this research with future possibilities resulting from this study.

Receptor Mediated Endocytosis

Figure 1.1. Receptor mediated endocytosis.

Receptor mediated endocytosis is initiated when the ligand binds to the receptor at the plasma membrane originating a series of intracellular events, which will allow the formation of coated vesicles. The ligand-receptor complex is internalized in endocytic vesicles (early and late endosomes) that ultimately separate the complex degrading the ligand in the lysosomal compartment and recycling the receptor (in some cases) back to the cell surface.

EGFR Structure

Figure 1.2. EGFR structure.

EGFR is composed in the extracellular (EC) region of two ligand-binding domains (LBD) and two cysteine-rich domains (CRD). The cytoplasmic tail, located inside the cell (IC), is composed of a tyrosine kinase domain (TKD) and a regulatory domain (RD). The extracellular and the cytoplasmic regions are divided by a transmembrane region (TMR).

Figure 1.3. EGFR signaling.

Major signaling cascades activated by EGFR. Once EGF binds to the receptor, Ras gets activated through the Grb2/SOS complex promoting the activation several signaling cascades including the Ral (Ras related protein)/PLD (phospholipase D), PI3K/ Akt, NORE1 (novel Ras effector 1)/MST (mammalian Sterile20-like), P190RhoGAP/ROCK (Rho-associated, coiled-coil containing protein kinase), PLC/Rap and Raf/Erk.

Insulin Receptor Structure

Figure 1.4. Insulin receptor structure.

IR is composed of a ligand-binding domain in the extracellular region (EC) made up of two large leucine-rich domains (L1 and L2), a cysteine-rich domain (CR) and two fibronectin type domains (Fn₀ and Fn₁). The cytoplasmic tail is composed of a tyrosine kinase domain (TKD) and the carboxy-terminal tail (CT) located inside the cell (IC). The extracellular and the cytoplasmic regions are divided by a transmembrane region (TMR).

Insulin Receptor Signaling

Figure 1.5. Insulin receptor signaling.

Major signaling cascades activated by IR. Once insulin binds to the receptor, Ras gets activated through the Grb2/SOS complex promoting the activation several signaling cascades such as the phosphorylation of Erk.

TrkA (Neurotrophic tyrosine kinase receptor type 1) Structure

Figure 1.6. TrkA structure.

TrkA is composed of a cysteine-rich (CR), a leucine-rich (L1) and a immunoglobulin-like (Ig-like) domain in the extracellular region (EC). The cytoplasmic tail is composed of the tyrosine kinase domain (tyr 490, 674 and 675) and the carboxy-terminal tail inside the cell (IC). The extracellular and the cytoplasmic regions are divided by a transmembrane region.

Figure 1.7. TrkA signaling.

Major signaling cascades activated by TrkA. Once NGF binds to the receptor, two main pathways get activated: PI3K/Akt/BAD and Ras, promoting the activation several signaling cascades such as Mapk and BCL2.

Figure 1.8. Ras signaling.

Major signaling cascades activated by Ras. Once the ligand binds to the receptor, Ras gets activated through the Grb2/SOS complex promoting the activation several signaling cascades including the Ral (Ras related protein)/PLD (phospholipase D), PI3K/ Akt, NORE1 (novel Ras effector 1)/MST (mammalian Sterile20-like), P190RhoGAP/ROCK (Rho-associated, coiled-coil containing protein kinase), PLC/Rap and Raf/Erk. Some Ras-GTP effectors compete for binding, such as Rin1 with PI3K and Raf.

Figure 1.9. Rab5/ Ras cycle.

Rab5 and Ras GTPases cycle between its inactive form (bound to GDP) and its active form (bound to GTP). Guanine nucleotide exchange factors (GEFs) promote the GDP-GTP exchange, while GTPase activating proteins (GAPs) promote GTP hydrolysis.

Figure 1.10. Rab5 GAPs proteins.

Proteins that promote GTP hydrolysis, inactivating Rab5. All of them share a TBC (Tre-2, Bub2 and Cdc16) domain responsible of the GTPase activity. RabGAP5 is composed of a TBC, SH3 (Src Homology 3) and RUN domains. TBC-2 is composed of PH (pleckstrin homology), CC (coiled coil), THR and TBC domains.

Figure 1.11. Rab5 GEFs proteins.

Proteins that promote GDP-GTP exchange, activating Rab5. All of them share a Vps9 domain responsible for the Rab5 activation. Rin1 is composed of four domains. The N-terminus contains the SH2 (Src Homology 2) and PR (proline rich) domains. The Vps9 (vacuolar protein sorting 9) and the RA (Ras association) domains are located at the C-terminus. Rin1 variant lacks some amino acids on the Vps9 domain. Rin2 and Rin3 are composed of the same domains as Rin1, but they differ in the number of repeats of the PR domain. Alsln is composed of RL (RCC1-like domains), DH, PH (pleckstrin homology), MORN motifs and Vps9 domains. Rabex-5 is composed of ZnF (Zinc finger), MIU (motif interacting with Ubiquitin), CC (coiled coil) and PR domains. Varp contains the Vps9a and two ankyrin domains. Rap6 is composed of Ras GAP, PH and Vps9 domains.

Figure 1.12. Rin1 domains.

Rin1 is composed of four domains. The N-terminus contains the SH2 and PR domains. The Vps9 and the RA domains are located at the C-terminus.

Figure 1.13. Rin protein family.

This protein family is composed of three members named Rin1, Rin2 and Rin3. All members are composed of the same domains: SH2, proline-rich (PR), Vps9 and a RA domain. They differ in the number of repeats of the PR domain.

Figure 1.14. Adipogenesis signaling.

Selective activation of key proteins on the different days of adipocyte differentiation. Adipogenesis is the result of specific activation of early (days 1 and 2), intermediate (days 3 and 4) and late genes (days 5-9) as showed above.

Figure 1.15. Transcription factors involved in adipogenesis.

Signaling cascades involved in fat cell formation. Insulin activate the IR and the Ras and PI3K signaling cascades. 1-Methyl-3-Isobutylxanthine (IBMX) activates C/EBP β and dexamethasone (DEXA) is responsible for the activation of C/EBP δ . All together activate C/EBP α and PPAR γ , the key adipogenic markers of adipogenesis.

II. MECHANISM OF INTERACTION BETWEEN RIN1 AND RTKS AND ITS EFFECTS TOWARD THE ACTIVITY OF RAB5 AND RAS.

Tyrosine kinase receptors are membrane-bound receptors that get internalized through receptor-mediated endocytosis. These receptors share a common structure: an extracellular region responsible for the ligand interaction, a hydrophobic region that transverses the lipid bilayer, and a cytoplasmic tail that interacts with molecules inside the cell. Through the extracellular region, the receptor gets activated upon ligand binding. The ligand binding leads to autophosphorylation of the cytoplasmic tail of specific tyrosine residues, which then serve as docking sites for proteins containing an SH2 domain. The interaction between receptor cytoplasmic tail and SH2-containing proteins may represent one of the several mechanisms of the signal transduction regulation. Thus, the aim of this chapter is to determine key residues of Rin1 required for the interaction with tyrosine kinase receptors such as NGFR, EGFR, and IR and how this interaction affects several downstream signaling pathways.

Interaction between Rin proteins and RTKs.

Ras interference 1 interacts with activated EGFR through its SH2 domain (Barbieri et al., 2003). Key amino acids in this domain interact with the phosphorylated tyrosine residues in the cytoplasmic tail of the activated receptor.

To study the interaction of Rin1 and EGFR two approaches were carried out: 1- immunoprecipitation and 2- GST-pulldown. Each one of these assays is described in the Material and Methods section.

First, an immunoprecipitation assay was carried out by utilizing several Rin1 constructs: Rin1:WT as well as individual Rin1 domains named Rin1:R2 (which contains SH2 and proline rich domains) and Rin1:R3 (which contains Vps9 and RA domains) (Figure 3.3).

Second, a GST-pulldown was carried out by preparing cell lysates from stimulated (+EGF) and non-stimulated (-EGF) NR6 fibroblasts overexpressing EGFR, and the following GST-labeled purified proteins: Rin1 (SH2:WT), Rin1 (SH2:H120L) and Rin1 (SH2:Y121F). Glutathione S-transferase (GST) alone was used as negative control in these experiments. Figure 2.1 shows a specific interaction of EGFR with Rin1:WT and Rin1:R2 (Figure 2.1A,B), but not Rin1:R3 (Figure 2.1 C). These results suggest that the SH2 domain of Rin1 recognizes phosphorylated tyrosine residues on the receptor cytoplasmic tail, which is present both in the Rin1:WT and Rin1:R2 constructs, but not in Rin1:R3.

Previous studies showed the important role of arginine 94 (Rin1:R94A) of Rin1 in the interaction with EGFR (Hu et al., 2008). Figure 2.2 shows the interaction of EGFR and the GST-tagged Rin1:SH2 domain (WT and mutants). This time, two other point mutations were studied: Rin1:H120L and Rin1:Y121F. It can be observed that none of these mutants affected the Rin1/EGFR.

To study the interaction of Rin1 and IR different techniques were utilized as described in the Material and Methods section. The same as with EGFR, an immunoprecipitation assay was used to study the Rin1-IR interaction. Figure 2.3 shows that there is a specific interaction with the phosphorylated receptor (+insulin) with Rin1:WT (Figure 2.3A) and Rin1:R2 (Figure 2.3B) (which contains

the SH2 domain). Surprisingly, there is also a strong interaction with the Rin1:R3 construct (Figure 2.3C). To further confirm this interaction, the yeast two hybrid method was used. Figure 2.4 shows the interaction between the IR tail and Rin1:R3. It also showed that Rin1:WT, as well as Rin1:R2, interact with the tail of the receptor as expected.

Finally, a GST-pulldown assay was carried out using lysates from stimulated (+insulin) and non-stimulated (-insulin) NIH3T3 fibroblasts overexpressing IR, and the following GST-tagged purified proteins: Rin1 (SH2), Rin1 (SH2:W69E), Rin1 (SH2:A76E), Rin1 (SH2:R94A), (SH2:H120L), (SH2:Y121F) and Rin1 (SH2:Y148F). Mutations in the SH2 domain of Rin1 were made on key amino acids that may play an important role in the interaction with RTKs. Figure 2.5A shows the Western blot and quantification of Rin1:WT, Rin1:W69A, and Rin1:A76E. Figure 2.5B shows the IR interaction with all mutants of the SH2 domain of Rin1. From the six mutants two of them have the most inhibitory effect (Rin1:A76E, Rin1:Y121F), while mutant Rin1:Y148F showed less inhibitory effect when compared to SH2:WT. Mutants W69E, R94A and Rin1:H120L have no effect at all in the interaction with the active IR.

The Rin1-NGFR interaction was analyzed by doing a GST-pulldown assay (as described in the Material and Methods section) using cell lysates from stimulated (+NGF) and non-stimulated (-NGF) PC12 cells, which overexpress the TrkA receptor. Figure 2.6 shows a clear interaction of the receptor with Rin1 upon NGF stimulation.

In summary, these results confirm that all three receptors interact with Rin1 upon stimulation through the SH2 domain, which recognizes and binds to the phosphorylated tyrosines in the cytoplasmic tail of the activated receptor. Mutations on this domain have been related to different types of diseases such as leukemia (Cazzaniga et al., 1999), autoinflammatory disease (Zhou et al., 2012), diabetes (Marion et al., 2002), and cancer (Friedman et al., 1993). Surprisingly, just the IR, and neither EGFR nor NGFR, interacts with the Vps9 domain of Rin1. The Vps9 domain is responsible for the binding and further activation of Rab5 by promoting GDP to GTP exchange.

Signaling regulation by Rin proteins.

The next section describes 1- how these interactions affect the activity of key endocytic and signaling proteins such as Rab5 and Ras, respectively, and 2- how they affects major biological processes such as cell differentiation.

Upon activation, RTKs initiate a cascade of protein-protein interactions that modulate the receptor internalization and signaling through the activation of the key GTPases Rab5 and Ras, respectively. Rab5 is responsible for the clathrin-coated vesicle transportation from plasma membrane to early endosomes and its further homotypic early endosome docking and fusion (Barbieri et al., 1994; Bucci et al., 1992; Gorvel et al., 1991; Li et al., 1994; Zerial and McBride, 2001). Receptor activation promotes recruitment and activation of Rab5, as was demonstrated for EGFR (Barbieri et al., 2000). Rab5 is inactivated by the interaction with GAPs proteins, such as RabGap5 (Haas et al., 2005), RN-tre (Albert et al., 1999), and TBC-2 (Chotard et al., 2010), by promoting the

hydrolyzation of GTP into GDP. GEF proteins, such as Rabex5, Alsln2, Vps9p and Rin1, promote the exchange of GDP into GTP activating Rab5. All these proteins share a Vps9 domain, which is the one responsible for the activation (Carney et al., 2006).

Figure 2.7 shows Rab5 activation in EGF-stimulated cells overexpressing Rin proteins. Ras interference 1 and Rin3 promote Rab5 activation compared with control, while Rin2 barely promoted any Rab5 activation (Figure 2.7). Interestingly, it was observed that all Rin family members also stimulated Rab5 activity in non-stimulated cells (Figure 2.7).

In the case of IR, Rab5 activation shows a similar tendency as in EGF-stimulated cells. (Figure 2.8) shows an increase in Rab5-GTP in Rin1 overexpressing cells, and to a lesser extent, in cells overexpressing Rin2 and Rin3. Also observed was that overexpression of all Rin proteins stimulates Rab5 activity in non-stimulated cells (Figure 2.8).

Ras is a major signaling protein responsible of amplifying RTK signaling. Active Ras is important in cell differentiation and proliferation, and when it is overactive, it has been related to numerous types of cancer (Fernandez-Medarde and Santos, 2011). GTPase activating proteins (GAPs) and GEF proteins regulate Ras activity in the same way as Rab5, or any other GTPase protein. Ras activation is initiated by the activation of the receptor. Once the receptor is activated, an adaptor protein (GRB2) will bind through its SH2 domain to a phosphorylated tyrosine residue in the cytoplasmic tail of the receptor. GRB2 also contains an SH3 domain, which will bind and activate SOS, a GEF, and

promote the GDP-GTP exchange activating Ras (Olivier et al., 1993). But Ras signaling regulation is much more complex than that. Proteins containing RA domain, such as Rin1, serve as Ras effectors (Ponting and Benjamin, 1996). These effectors directly interact with the active form of Ras, without having any effect on the rate of GTP hydrolysis. Even though Ras effectors do not inactivate Ras, they can have an impact in signaling cascades by competing with other effectors. This aim studied the effect of the Rin proteins in Ras and its signaling cascades activation.

Figure 2.9 shows that there is no effect in Ras activity upon EGF stimulation if Rin1 is overexpressed when compared to control cells. As mentioned above, Rin proteins have a domain (RA) that interacts with the active form of Ras (Barbieri et al., 2003). This interaction may have an effect in one or more signaling pathways activated by Ras.

To study the effect of Rin overexpression in Ras-activated signal transduction several signaling proteins were examined. Figure 2.10 shows the effect of Rin1 overexpression in NR6 cells stimulated with EGF. As expected, the absence of EGF did not stimulate any of the activities analyzed (Figure 2.10A-E). A small inhibition of the tyrosine phosphorylation of EGFR was observed in cells expressing Rin1 (Figure 2.10E). Akt, Erk, and Junk activities were dramatically blocked in cells expressing Rin1 as compared with control-stimulated cells (Figure 2.10A,B and D). In contrast, p38 activity was not affected (Figure 2.10C). Rin2 overexpression in EGF-stimulated NR6 cells generated slightly different results. Figure 2.11 shows a significant inhibition of phosphorylated Akt (Figure

2.11B), while Erk (Figure 2.11A) activation seems to be enhanced. The activation of p38 (Figure 2.11C), similar to Rin1 overexpression, is not affected by the overexpression of Rin2. In the case of Rin3 overexpression, there is an inhibition in phosphorylated Erk (Figure 2.12A) and Akt (Figure 2.12B). Once again, there is no effect on p38 phosphorylation (Figure 2.12C), just as in the case of Rin1 and Rin2 overexpression (Figure 2.12) upon EGF-stimulation.

The effect of Rin proteins overexpression was also studied in NIH3T3 fibroblast overexpressing IR. Figure 2.13 shows the effect of Rin1 overexpression in the Ras-activated signaling cascades. As in the EGFR overexpressed cells, Rin1 inhibits Erk (Figure 2.13A) and Akt (Figure 2.13B) phosphorylation upon insulin stimulation. In this case, opposite to what is observed upon EGF stimulation, Rin1 did not block p38 (Figure 2.13C) and IR (Figure 2.13D) phosphorylation upon stimulation. A similar tendency was observed when Rin2 is overexpressed. Figure 2.14 shows an inhibitory effect on Erk (Figure 2.14A) and Akt (Figure 2.14B) activation, while it promotes p38 (Figure 2.14C) phosphorylation upon insulin stimulation. In the case of Rin3 overexpression, Figure 2.15 shows a similar inhibitory effect on Erk (Figure 2.15A) observed for Rin1 and Rin2, but this time there is no significant effect on Akt (Figure 2.15B) or p38 (Figure 2.15C) activation.

To obtain a better understanding of the effect of Rin1 in IR signaling, several residues of each domain of Rin1 were mutated and their effects in insulin driven signaling pathways were studied. The following residues were mutated: Rin1:R94A (SH2 domain), Rin1:Y561F (Vps9 domain), Rin1:T580A (Vps9

domain) and Rin1:R629A (RA domain). Interestingly, arginine 94 is important in the interaction of Rin1 with activated EGFR (Hu et al., 2008). Figure 2.16 shows that Rin1:R94A mutant has no effect on IR phosphorylation (Figure 2.16A), it reverses the inhibitory effect of Rin1:WT (Figure 2.16B), and more importantly, it has a greater inhibitory role than the wild type protein in Akt activation (Figure 2.16C). It has been shown that tyrosine 561 is necessary for the proper activation of Rab5, and it is involved in the interaction with active Ras (Galvis et al., 2009). Figure 2.17 shows that Rin1:Y561F mutant has no effect in IR phosphorylation (Figure 2.17A), but it reverses the inhibitory effect caused by Rin1:WT in both Erk (Figure 2.17B) and Akt (Figure 2.17C) activity. Figure 2.18 shows that Rin1:T580A mutant, which blocks Rab5 activation but not Ras binding (Galvis et al., 2009), enhances the phosphorylation of the receptor (Figure 2.18A) and reverses the inhibitory effect in both Erk (Figure 2.18B) and Akt (Figure 2.18C) phosphorylation. Lastly, Rin1:R629A mutant, which affects the interaction with Ras, enhances the phosphorylation of IR (Figure 2.19A) and Akt (Figure 2.19C) compared to Rin1:WT, while it has a greater inhibitory effect on Erk (Figure 2.19B) activity. Interestingly, all mutants promote the activation of p38 in non-simulated cells; in some cases this activation is greater than the one observed in insulin stimulated cells (Figures 2.16- 2.19D).

Figure 2.1. Immunoprecipitation assay between Rin1 (WT and domains) and the EGF receptor.

NR6 fibroblasts overexpressing EGFR, in the presence (+EGF) or absence (-EGF) of 100ng/ul EGF, were lysed and immunoprecipitated as explained in Material and Methods. Stimulated and non-stimulated cell lysates were immunoprecipitated with an anti-EGFR antibody, and the presence of Rin1 (**A**), R2 (**B**) or R3 (**C**) bound to the receptor was visualized using anti-Rin1 antibody. Rin1 and EGFR present in the total cell lysate (TCL) shows the total amount of protein added to the assay. Rin1 present in the IP sample represents the amount of protein bound to the receptor. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to EGF-stimulated cells.

Figure 2.2. GST-pulldown assay between EGFR receptor and Rin1 mutants.

NR6 cells overexpressing EGFR, in the presence (+EGF) or absence (-EGF) of 100ng/ul EGF, were lysed and incubated with GST-fused lysates of the different Rin1 constructs, as explained in Material and Methods. In the presence of EGF, cell lysates were incubated with Rin1:H120L and Rin1:Y121F mutants, and the amount of Rin1 protein bound to the receptor was visualized using anti-EGFR antibody. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to EGF-stimulated cells.

Figure 2.3. Immunoprecipitation assay between Rin1 (WT and domains) and the insulin receptor.

NIH3T3 fibroblasts overexpressing IR, in the presence (+Ins) or absence (-Ins) of 100nM insulin, were lysed and immunoprecipitated as explained in Material and Methods. Stimulated and non-stimulated cell lysates were immunoprecipitated with anti-IR antibody, and the presence of Rin1 (**A**), R2 (**B**) or R3 (**C**) bound to the receptor was visualized using anti-Rin1 antibody. Rin1 and IR present in the total cell lysate (TCL) shows the total amount of protein added to the assay. Rin1 present in the IP sample represents the amount of protein bound to the receptor. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to insulin-stimulated cells.

Figure 2.4. Yeast two hybrid system using Rin1 constructs and the insulin receptor cytoplasmic tail.

Rin1, R2 (SH2 and proline rich domains) and R3 (Vps9 and RA domains) were used to do the yeast two hybrid system with the insulin receptor cytoplasmic tail as explained in Materials and Methods. The insulin receptor tail: kinase dead was used as negative control. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to IR: kinase dead (control).

Figure 2.5. GST-pulldown assay between the insulin receptor and Rin1 mutants.

Pure recombinant insulin receptor tail was phosphorylated in the presence of ATP, as explained in the Material and Methods section, prior to the binding to Rin1-SH2 mutants GST-fused lysates. The amount of Rin1 protein bound to the receptor was visualized using anti-IR antibody. **(A)** Western blot and quantification of the IR interaction with Rin1:SH2 WT, Rin1:W69A and Rin1:A76E. **(B)** Quantification of the IR interaction with the Rin1: SH2 mutants. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to Rin1:WT.

Figure 2.6. GST-pulldown assay between the NGF receptor and Rin1 mutants.

PC12 cells overexpressing TrkA, in the presence (+NGF) or absence (-NGF) of 100nM NGF, were lysed and incubated with GST-tagged lysates of Rin1:SH2 domain, as explained in Material and Methods. The amount of Rin1 protein bound to the receptor was visualized using anti-NGFR antibody. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to NGF-stimulated cells.

Figure 2.7. Rab5 activation in NR6 cells overexpressing EGFR and Rin proteins.

Rab5 activation was measured as described in Materials and Methods in NR6 control fibroblasts and cells overexpressing Rin1, Rin2 and Rin3 in the presence (+EGF) or absence (-EGF) of 100ng/μl EGF. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean±S.E.M. of three independent experiments. * $P < 0.05$ by Student's t-test compared to EGF-stimulated cells.

Figure 2.8. Rab5 activation in NIH3T3 cells overexpressing Rin1.

Rab5 activation was measured as described in Materials and Methods in NIH3T3 control fibroblasts and cells overexpressing Rin1, Rin2 and Rin3 in the presence (+Ins) or absence (-Ins) of 100nM insulin. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to insulin-stimulated cells.

Figure 2.9. Ras activation in NR6 cells overexpressing Rin1.

NR6 cells expressing GFP (circle-blue) and Rin1:WT (square-red) were lysed and incubated with the GST-fused Ras binding domain of Raf. The pull-downs were subjected to SDS-PAGE and analyzed by Western blotting using an anti-Ras antibody. Relative Units (% of maximum) of proteins were determined by densitometry using the ratio of active Ras to total-Ras. Data represent the mean $\pm$ S.E.M. of three independent experiments.

Figure 2.10. EGFR signaling in NR6 cells overexpressing Rin1.

NR6 fibroblasts control and cells overexpressing Rin1 were stimulated in the presence (+EGF) or absence (-EGF) of 100ng/μl EGF and the effect on phosphorylation of Erk (A), Akt (B), p38 (C), JunK (D), and EGFR (E) was measured on total cell lysates using the appropriate antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean±S.E.M. of three independent experiments. * $P < 0.05$ by Student's t-test compared to EGF-stimulated cells. ** $P < 0.05$ by Student's t-test compared to Rin1 overexpressing/ EGF-stimulated cells.

Figure 2.11. EGFR signaling in NR6 cells overexpressing Rin2.

NR6 fibroblasts control and cells overexpressing Rin2 were stimulated in the presence (+EGF) or absence (-EGF) of 100ng/ μ l EGF and the effect on phosphorylation of Erk (**A**), Akt (**B**) and p38 (**C**), was measured on total cell lysates using the appropriate antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to EGF-stimulated cells. ** P <0.05 by Student's t-test compared to Rin2 overexpressing/ EGF-stimulated cells.

Figure 2.12. EGFR signaling in NR6 cells overexpressing Rin3.

NR6 fibroblasts control and cells overexpressing Rin3 were in the presence (+EGF) or absence (-EGF) of 100ng/ μ l EGF and the effect on phosphorylation of Erk (**A**), Akt (**B**) and p38 (**C**), was measured on total cell lysates using the appropriate antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to EGF-stimulated cells. ** P <0.05 by Student's t-test compared to Rin3 overexpressing/ EGF-stimulated cells.

Figure 2.13. IR signaling in NIH3T3 cells overexpressing Rin1.

NIH3T3 fibroblasts control and cells overexpressing Rin1 were stimulated in the presence (+Ins) or absence (-Ins) of 100nM insulin and the effect on phosphorylation of Erk (A), Akt (B), p38 (C), and IR (D) was measured on total cell lysates using the appropriate antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P < 0.05 by Student's t-test compared to insulin-stimulated cells. ** P < 0.05 by Student's t-test compared to Rin1 overexpressing/ EGF-stimulated cells.

Figure 2.14. IR signaling in NIH3T3 cells overexpressing Rin2.

NIH3T3 fibroblasts control and cells overexpressing Rin2 were stimulated in the presence (+Ins) or absence (-Ins) of 100nM insulin and the effect on phosphorylation of Erk (**A**), Akt (**B**), and p38 (**C**) was measured on total cell lysates using the appropriate antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to insulin-stimulated cells. ** P <0.05 by Student's t-test compared to Rin2 overexpressing/ EGF-stimulated cells.

Figure 2.15. IR signaling in NIH3T3 cells overexpressing Rin3.

NIH3T3 fibroblasts control and cells overexpressing Rin3 were stimulated in the presence (+Ins) or absence (-Ins) of 100nM insulin and the effect on phosphorylation of Erk (**A**), Akt (**B**), and p38 (**C**) was measured on total cell lysates using the appropriate antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to insulin-stimulated cells. ** P <0.05 by Student's t-test compared to Rin3 overexpressing/ EGF-stimulated cells.

Figure 2.16. IR signaling in NIH3T3 cells overexpressing Rin1:R94A mutant. NIH3T3 fibroblasts overexpressing Rin1:WT (used as control) and Rin1:R94A were stimulated in the presence (+Ins) or absence (-Ins) of 100nM insulin and the effect on phosphorylation of IR (A) Erk (B), Akt (C), and p38 (D) was measured on total cell lysates using the appropriate antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to insulin-stimulated cells. ** P <0.05 by Student's t-test compared to Rin1:WT overexpressing/ EGF-stimulated cells.

Figure 2.17. IR signaling in NIH3T3 cells overexpressing Rin1: Y561F mutant.

NIH3T3 fibroblasts overexpressing Rin1:WT (used as control) and Rin1:Y561F were stimulated in the presence (+Ins) or absence (-Ins) of 100nM insulin and the effect on phosphorylation of IR (A) Erk (B), Akt (C), and p38 (D) was measured on total cell lysates using the appropriate antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to insulin-stimulated cells. ** P <0.05 by Student's t-test compared to Rin:WT overexpressing/ EGF-stimulated cells.

Figure 2.18. IR signaling in NIH3T3 cells overexpressing Rin1: T580A mutant.

NIH3T3 fibroblasts overexpressing Rin1:WT (used as control) and Rin1:T580A were stimulated in the presence (+Ins) or absence (-Ins) of 100nM insulin and the effect on phosphorylation of IR (A) Erk (B), Akt (C), and p38 (D) was measured on total cell lysates using the appropriate antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to insulin-stimulated cells. ** P <0.05 by Student's t-test compared to Rin1:WT overexpressing/ EGF-stimulated cells.

Figure 2.19. IR signaling in NIH3T3 cells overexpressing Rin1:R629A mutant.

NIH3T3 fibroblasts overexpressing Rin1:WT (used as control) and Rin1:R629A were stimulated in the presence (+Ins) or absence (-Ins) of 100nM insulin and the effect on phosphorylation of IR (**A**) Erk (**B**), Akt (**C**), and p38 (**D**) was measured on total cell lysates using the appropriate antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to insulin-stimulated cells. ** P <0.05 by Student's t-test compared to Rin1:WT overexpressing/ EGF-stimulated cells.

III. THE ROLE OF RIN1 (AND RIN1-LIKE MOLECULES) IN SIGNALING TRANSDUCTION PATHWAYS INVOLVED IN CELL DIFFERENTIATION.

Cell proliferation, differentiation, and apoptosis are exceptionally similar processes in both normal and diseased cells. However, they show unique traits. Cell proliferation is a complex molecular mechanism that involves the regulation of genes. These genes are required for cell division cycle, which is an orderly and unidirectional transition from one cell cycle phase to the next. Cell differentiation is a process in which a cell develops into certain type upon specific stimulation. Cell differentiation changes a cell's size, shape, intracellular metabolism, and more important it is sensitive to external signals. These changes are largely the result of highly controlled and regulated gene expression upon stimulation. Several *in vitro* differentiation systems have been described. Here, two in vitro differentiation systems, 3T3L1 preadipocytes and PC12 cells, are studied.

Adipogenesis is the formation of adipocytes (fat cells) from preadipocytes. Different types of hormones and signaling cascades, including insulin, regulate this process. To study adipogenesis *in vitro*, preadipocytes cells are exposed to an induction cocktail composed of insulin, dexamethasone (dexamethasone) and 1-Methyl-3-Isobutylxanthine (IBMX). The induction cocktail activates adipogenic factors C/EBP β and δ , which are responsible for the activation of C/EBP α and PPAR γ . The last ones are key regulators of adipogenesis; they are responsible for the morphological and transcriptional changes in adipocytes. Ras interference 1, as explained above, is a multifunctional protein involved both in the internalization

and signal transduction of IR. In the present study 3T3-L1 preadipocytes cells were used to determine the effects of Rin1 (its domains and Rin1-like molecules) during the differentiation process. Several Rin1 point mutations as well as Rin1 deletion mutants were created on key amino acids that affect the function of each domain in order to analyze their effects on preadipocyte differentiation *in vitro*.

Ras interference 1 is part of a protein family composed of two other members: Rin2 and Rin3. The three of them share a highly conserved Vps9 domain that activates Rab5. Figure 3.1 shows the endogenous expression levels of Rab5 and Rin1 in days 1, 3, 5 and 10 of differentiation. The expression of Rab5 increases until day 5, and then there is a decrease up to day 10 (Figure 3.1A). In the case of Rin1, it highly expressed in fibroblasts, and upon stimulation, there is a constant decrease (Figure 3.1B). To study the effect of Rin proteins in 3T3-L1 differentiation, stable cell lines were done, differentiated for ten days and the lipid droplet formation quantified as explained in Materials and Methods. Figure 3.2A shows that individual overexpression of Rin proteins inhibited adipocyte differentiation. Ras interference 1 showed the greater inhibitory effect. In contrast, when Rin1 is depleted by RNAi, the inhibitory effect was not observed (Figure 3.2B).

To determine which specific domain of Rin1 is responsible for the inhibitory effect on adipocyte differentiation, the N- (Rin1:R2), C-terminus (Rin1:R3) and each individual domain were cloned and overexpressed in 3T3-L1 cells (Figure 3.3). Following the protocol described in Material and Methods section, the lipid droplet formation was quantified at day 10 of differentiation and

is showed in Figure 3.2C. As expected, the C-terminus (Rin1:R3) and the Vps9 domain alone (Rin1:R4) were enough to produce the inhibitory effect. Surprisingly, the same was observed for each individual domain (SH2 and RA) and for the N-terminus. All of them had a similar inhibitory effect as Rin1:WT.

To confirm the morphological effects and lipid quantification, expression of key adipogenic markers (C/EBP α and PPAR γ) and signaling proteins (Erk and Akt) were studied on day 10 of differentiation of all cell lines overexpressing Rin1 and its domains. Figure 3.4 shows that the expression of both C/EBP α (Figure 3.4A) and PPAR γ (Figure 3.4B) is inhibited compared to control cells, supporting the morphological and lipid quantification data. The expression of PPAR γ is dramatically inhibited, which explains why the cells overexpressing Rin1 resemble a fibroblast-like shape instead of an adipocyte one. PPAR γ is known to be the master regulator of adipocyte differentiation; its expression in the cell is absolutely necessary in order for adipogenesis to occur. On the other hand, activation of important signaling proteins such as Erk (Figure 3.4C) and Akt (Figure 3.4D), does not seem to be affected by the overexpression of Rin1. These observations may suggest that the mechanism used by Rin1 to inhibit adipogenesis is independent from the Erk and PI3K signaling pathways. Figure 3.5 shows that the different Rin1 domains have the same inhibitory tendency on the adipogenic markers as Rin1:WT. Again, PPAR γ (Figure 3.5B) is the one inhibited the most, even though the higher inhibition is observed by the expression of Rin1:WT, but not by the expression of any specific Rin1 domain.

In an effort to elucidate which Rin1 domain(s) is/are required in the inhibition of differentiation, the following key amino acids (for the proper functioning) of the SH2, Vps9 and RA domains were mutated and overexpressed as described in Material and Methods section: Rin1:R94A, Rin1:Y561F, Rin1:R94A+Y561F, Rin1:T580A and Rin1:R629A. Each mutant is able to reverse the inhibitory effect shown by its domain, as it is shown in Figure 3.6. The same is observed in the expression of the key adipogenic markers: the Rin1 mutants are almost able to restore the normal expression levels for C/EBP α and PPAR γ (Figure 3.7A and B, respectively). The effect of the Rin1 mutants supports previous morphological and biochemical data that show that all the domains play an important role in the inhibitory effect that Rin1 has in adipocyte differentiation (Figure 3.3).

In order to understand the molecular mechanism by which Rin1 inhibits *in vitro* differentiation of 3T3-L1 cells, controls as well as Rin1-overexpressing cells were differentiated and the activation of CREB was analyzed at days 1, 3, 5 and 10 of treatment. Figure 3.8 shows that activation of CREB increases during differentiation of control cells, opposite to what is seen in Rin1-overexpressing cells: a decrease during the differentiation treatment. Even though these data is complex to explain, it is the only difference in signaling that has been found so far when Rin1 is overexpressed, so it may help elucidate the exact mechanism by which Rin1 inhibit 3T3-L1 preadipocyte differentiation.

To investigate whether Rin1 has a similar inhibitory effect in other cell differentiation systems, a second differentiation system was studied. PC12 cells

overexpress the TrkA receptor and respond to NGF differentiating into neuron-like cells with neurite projections. PC12 cells overexpressing Rin1, Rin2 and Rin3 were exposed to NGF for ten days and the neurite projections were measured.

Cells overexpressing the Rin proteins were NGF stimulated and the neurite growth quantified 10 days later as described in Materials and Methods. Figure 3.9 shows that any of the Rin proteins have a major effect on PC12 differentiation (percentage of differentiated cells: Control 57.23%, Rin1: 47.62%, Rin2 56.5% and Rin3 50.5%). Overexpression of Rin1, Rin2 or Rin3 has no effect on the amount of cells that actually commit to differentiation, or in the length of the neurite projections. The fact that there seems to be no major effect by Rin1 in PC12 differentiation compared to its regulatory role in 3T3-L1 differentiation, may suggest a receptor and cell specific role of Rin1.

Figure 3.1. Rab5/ Rin1 endogenous expression during 3T3-L1 differentiation.

3T3-L1 preadipocytes were induced to differentiate by induction media into adipocytes as explained in Material and Methods. Total protein extracts were prepared at day 0, 3, 5, and 10. The proteins were subset to 12% SDS PAGE electrophoresis, blotted to a nitrocellulose membrane, and probed with antibodies specific to Rab5 (**A**) and Rin1 (**B**). Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean±S.E.M. of three independent experiments. * $P<0.05$ by Student's t-test.

Figure 3.2. Lipid droplet quantification in cells overexpressing Rin proteins and Rin1 domains.

(A) 3T3-L1 preadipocytes overexpressing Rin1, Rin2 and Rin3 proteins were differentiated for 10 days as described in Material and Methods. Lipid quantification was done and compared to control cells. Results were represented as Relative Units (relative lipid contents). The data represent the mean $\pm$ SEM of 3 independent experiments. **(B)** 3T3-L1 preadipocytes were transfected with Rin1 RNAi for different period of times during the differentiation process as described in Material and Methods. Lipid quantification was done on day 10 and compared to control cells. Results were represented as Relative Units (relative lipid contents). The data represent the mean $\pm$ SEM of 3 independent experiments. **(C)** 3T3-L1 preadipocytes overexpressing Rin1 domains were differentiated as explained above. Lipid quantification is compared to control and cells overexpressing Rin1. Results were represented as Relative Units (relative lipid contents). The data represent the mean $\pm$ SEM of 3 independent experiments. * $P < 0.05$ by Student's t-test compared to control cells.

Figure 3.3. Different constructs of the domains of Rin1.

R2 represents the N-terminus of the protein containing the SH2 and PR domains. R3 represents the C-terminus of Rin1 containing the Vps9 and RA domains. R4 represents the Vps9 domain, and R5 the RA domain.

Figure 3.4. Adipogenic markers expression in cells overexpressing Rin1.

3T3-L1 preadipocytes overexpressing Rin1:WT were differentiated for 10 days and total protein extracts were prepared as described in Material and Methods. Total protein extracts were subset to 10% SDS-PAGE electrophoresis, blotted to a nitrocellulose membrane and probed against the following antibodies: **(A)** C/EBP α , **(B)** PPAR γ , **(C)** P-Erk and T-Erk, **(D)** P-Akt and T-Akt. Results were represented as Relative Units (relative lipid contents). Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to control cells.

Figure 3.5. Adipogenic markers expression in cells overexpressing the different Rin1 domains.

3T3-L1 preadipocytes overexpressing Rin1 and the different domains were differentiated and total protein extracts were obtained as explained above. The nitrocellulose membrane was probed against **(A)** C/EBP α , **(B)** PPAR γ and **(C)** P-Erk. Results were represented as Relative Units (relative lipid contents). Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to control cells.

Figure 3.6. Lipid droplet quantification in cells overexpressing Rin1 mutants.

3T3-L1 preadipocytes overexpressing Rin1:WT and the following mutants: R94A, Y561F, R94A+Y561F, T580A and R629A, were differentiated for 10 days as described in Material and Methods. Lipid quantification was done and compared to control cells. Results were represented as Relative Units (relative lipid contents). The data represent the mean $\pm$ SEM of 3 independent experiments. * $P < 0.05$ by Student's t-test compared to control cells.

Figure 3.7. Adipogenic markers expression in cells overexpressing the different Rin1 mutants.

3T3-L1 preadipocytes overexpressing the different Rin1 mutants were differentiated for 10 days and total protein extracts were prepared as described in Material and Methods. Total protein extracts were subset to 10% SDS-PAGE electrophoresis, blotted to a nitrocellulose membrane and probed against the following antibodies: **(A)** C/EBPα, **(B)** PPARγ, **(C)** P-Erk and T-Erk. Results were represented as Relative Units (relative lipid contents). Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean±S.E.M. of three independent experiments. * $P<0.05$ by Student's t-test compared to control cells.

Figure 3.8. CREB activation in cells overexpressing Rin1 and control cells. 3T3-L1 control preadipocytes **(A)** and cells overexpressing Rin1 **(B)** were induced to differentiate by induction media into adipocytes as explained in Material and Methods. Total protein extracts were prepared at day 1, 3, 5, and 10. The proteins were subset to 12% SDS PAGE electrophoresis, blotted to a nitrocellulose membrane, and probed with antibodies specific to T-CREB and P-CREB. Results were represented as Relative Units (relative lipid contents). Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test.

Figure 3.9. Effect of Rin proteins in PC12 differentiation.

PC12 control cells and overexpressing Rin proteins were stimulated with 100ng/ μ l NGF and the neurite growth quantified 10 days later as described in Materials and Methods. **(A)** Percentage of differentiated cells. **(B)** Average branch length. **(C)** Average number of branches per cell. Pictures were taken at day 10 of differentiation for control cells **(D)**, Rin1 **(E)**, Rin2 **(F)** and Rin3 **(G)** overexpressing cells. * $P < 0.1$ by Student's t-test compared to control cells.

IV. EXAMINING THE EFFECT OF DEHYDROLEUCODINE IN DIFFERENTIATION OF ADIPOSE CELLS.

Sesquiterpene lactones are a large and structurally diverse group of plant second metabolites (Heinrich et al., 1998) with distinctive biological activities, including gastric cytoprotector effects (Penissi et al., 1998), anti-migraine (Beekman et al., 1997), antiviral and antimicrobial activities (Hayashi et al., 1996; Perry and Foster, 1995), anti-tumor (Robles et al., 1995) and neurotoxic effect (Cheng et al., 1992).

Sesquiterpene lactones are also blockers of smooth muscle contractility (Hay et al., 1994) aromatase activity (Blanco et al., 1997) and NF- κ B activation (Hegner et al., 1998; Lyss et al., 1998). Also, it has been found that sesquiterpene lactones inhibit the activation of cyclooxygenase and pro-inflammatory cytokines in macrophages (Hwang et al., 1996). Within the group of sesquiterpene lactones, helenalin, which occurs in the aerial portion of *Arnica Montana* L., was also found to block the hormonally induced Sky2 mRNA and Akt phosphorylation during early stages of adipocyte differentiation (Auld et al., 2006). Inhibitory activities have been principally linked to the α -methylene- γ -lactone function (Heinrich et al., 1998). However, the reduction of the α -methylene- γ -lactone limited its cytotoxicity effect without affecting the anti-proliferative and anti-aromatase activity (Blanco et al., 1997).

Dehydroleucodine (DhL) is a sesquiterpene lactone of the guaianolide group, which also contains a α -methylene- γ -lactone ring in its molecule. It was first isolated from *Lidbeckia pectinata* (Bohlmann and Zdero, 1972). The aerial

parts of *Artemisia douglasiana* Besser are also rich in DhL (Giordano et al., 1990). Chloroform extracts of the air-dried aerial parts of *Artemisia douglasiana* showed significant gastric cytoprotective activity (Giordano et al., 1992). In addition, DhL inhibited cell proliferation (Polo et al., 2007) and growth of *Trypanosome cruzi* in culture (Breggio et al., 2000). Recent studies also showed the antiproliferative effects of DhL on B16 mouse melanoma cells, without having an effect on the normal Melan-A mouse melanocytes (Priestap et al., 2012). Furthermore, it has been proved that the exposure of DhL (either transient or continuous) to cancer cells inhibits proliferation and promotes apoptosis of human cancer cells by the activation of p53 and the DNA damage control mechanism, in a dose dependent manner (Costantino et al., 2013).

To examine the potential role of DhL on the differentiation of preadipocytes, cells were incubated with induction media in the presence of various concentration of DhL. Figure 4.1A shows that the addition of DhL inhibited the lipid content in a dose-dependent manner with an IC₅₀ of 6μM. Furthermore, the experimental conditions demonstrate that the addition of DMSO (0.2%) exclusively does not hinder 3T3-L1 preadipocytes differentiation.

To examine the effect of DhL on the viability of 3T3-L1 preadipocytes, cells were incubated with induction media in the presence of 8μM DhL for 24hrs (Day 1) or throughout the entire 9 days of differentiation (Day 9). Cell viability was measured using the MTT colorimetric assay as described in Material and Methods section. Figure 4.1A shows that the addition of DhL blocked the differentiation of 3T3-L1 preadipocytes whereas cell viability was not affected as

compared with DMSO or untreated cells (Figure 4.1B). However, at higher DhL concentrations ($>10\mu\text{M}$), 3T3-L1 preadipocytes detached from the plate, which was coupled with a significant reduction in the MTT assay. These results suggest that, up to a maximum concentration of $10\mu\text{M}$, DhL has a strong inhibitory activity of 3T3-L1 preadipocytes differentiation without significant effect on cell viability.

Adipocyte differentiation can be also monitored by formation of intracellular lipid droplets (Rosen and Spiegelman, 2000). As described above, 3T3-L1 preadipocytes were cultivated and induced to differentiate into adipocytes with induction media in the absence or presence of $8\mu\text{M}$ DhL. At day 9, Oil Red O staining showed that an abundant number of lipid droplets suggesting a significant lipid accumulation in untreated differentiated cells. However, lipid droplets were not observed in untreated non-differentiated cells (compare Figure 4.2A and B). More importantly, formation of lipid droplets was inhibited by $8\mu\text{M}$ DhL treatment (Figure 4.2C). These observations were further supported with the quantitative measurement of lipid content by determining the absorbance at 540nm (Figure 4.1A). In addition, DhL treatment significantly inhibited adipogenic morphology (i.e., transition from a fibroblast-like shape to an increasingly rounded-up appearance with an accumulation of cytoplasmic lipid droplets; compare Figure 4.2B and C).

Given that DhL inhibited differentiation to 3T3-L1 preadipocytes, next it was considered whether DhL would inhibit triglyceride accumulation. Cells treated with induction medium in the presence of $8\mu\text{M}$ of DhL accumulated

roughly $31 \pm 5\%$ of the intracellular triglyceride contained in controls (Figure 4.2D). As expected, DMSO-treated cells did not affect the formation of triglyceride accumulation as compared with cells incubated with induction media alone.

Adipogenesis is a highly regulated process requiring coordinated expression and activation of key transcriptional factors and signaling molecules (Rosen and Spiegelman, 2000). To investigate whether DhL affects the expression of PPAR γ and C/EBP α , 3T3-L1 preadipocytes were incubated with induction media in the absence or presence of 8 μ M DhL, and then harvested at day 9 for Western blot analysis using anti-PPAR γ and C/EBP α antibodies. Figure 4.3A shows that the addition of DhL clearly attenuated the expression of PPAR γ and C-EBP α . The effect of DhL on the phosphorylation of Akt1 and Erk1/2 proteins was also examined. Surprisingly, it was found that the addition of DhL did not attenuate phosphorylation of Erk and Akt (Figure 4.3B and C). Furthermore, it was tested whether the addition of DhL affected the phosphorylation of AMPK α during 3T3-L1 preadipocytes differentiation. The data shows that phosphorylation of AMPK α (P-AMPK α) was not inhibited by the addition of DhL (Figure 4.3D). In contrast, DhL further enhanced phosphorylation of AMPK α (Figure 4.3D). It was also found that the level of total AMPK α appeared relatively constant in the presence or absence of DhL. Therefore, these results indicated that the level of phosphorylated AMPK α increased in the presence of DhL. Interestingly, both PPAR γ and C/EBP α are selectively expressed during the differentiation of 3T3-L1 preadipocytes. The levels of

expression of these transcription factors is undetectable in preadipocytes; however, expression increases two days after induction and they are expressed five days after the induction of differentiation (Rosen and Spiegelman, 2000). Consistent with these observations, it was also observed that the expression of PPAR γ and C-EBP α was significantly increased with the progression of the differentiation of 3T3-L1 preadipocyte (Figure 4.4A and B). However, DhL treatment at the concentration of 8 μ M significantly blocked the expression of PPAR γ and C-EBP α (Figure 4.4A and B). In addition, the differentiation of 3T3-L1 preadipocyte progressed with an increase of the phosphorylation status of AMPK α (Figure 4.4C). Treatment with DhL further significantly increased the phospho-status of AMPK α , suggesting that DhL also induced the activation of AMPK α (Figure 4.3C). Furthermore, the addition of DhL also inhibited, in a concentration dependent manner, the expression of FAS (Fatty acid synthase) (Figure 4.6). Fatty acid synthase is a key protein in the production of lipids, responsible for the synthesis reaction of long-chain fatty acids (Semenkovich, 1997). On the other hand, AMPK stimulates energy production promoting glucose transport and fatty acid oxidation, inhibiting lipogenesis and protein synthesis (Daval et al., 2006). Taken together, these data suggest that DhL selectively blocks the expression of FAS, PPAR γ and C-EBP α and also increases the phosphorylation of AMPK α during the differentiation of 3T3-L1 preadipocytes.

It is also possible that the extent of inhibition is dependent on the timing of DhL addition. For this purpose, 8 μ M DhL was added in discrete periods during differentiation (Figure 4.5A). A significant reduction in the differentiation of 3T3-L1 preadipocytes was observed when compared with DMSO-control cells upon early DhL addition, corresponding to days 1 or 3 of treatment (Figure 4.5A and B). However, this inhibitory effect was not apparent when DhL was added on day 5, 7 or at day 8 post-induction. These results indicate that DhL may affect early adipocyte gene expression during *in vitro* differentiation.

These results clearly show that the addition of DhL inhibited differentiation of 3T3-L1 preadipocytes in a dose-dependent manner without a significant effect on cell toxicity (Figure 4.1). However, it has been postulated that the non-saturated α -methylene- γ -lactone function of sesquiterpene lactones produces an unspecific toxic effect leading to cell death (Polo et al., 2007). Therefore, it has been investigated the effect of DH-DhL, which is a derivative of DhL lacking alkylating function, on the differentiation of 3T3-L1 preadipocytes.

Dehydroleucodine (Figure 4.7, compound 1) can be gently reduced with sodium borohydride to give the corresponding 11,13-dihydro derivative (classical nomenclature; (Giordano et al., 1990). Analysis of the reaction product (DH-DhL) by GC shows that two epimers (Figure 4.7B, compounds 2 and 3) are formed in different amounts (Figure 4.7B) due to the generation of a chiral center at C-11 of the molecule during reduction. The 11S- epimer (compound 2) is the major reaction product (Giordano et al., 1992). It is accompanied by the minor 11R- epimer (compound 3), and by traces of unreacted DhL (compound 1) (Figure

4.7B). In the following these epimers are denoted (11S)DH-DhL and (11R)DH-DhL, respectively, for simplicity. After separation by preparative HPLC, the epimers were obtained in pure form and confirmed by GC analysis (Figure 4.7C and D). They were studied, in parallel with DhL, for their effect on the differentiation of 3T3-L1 preadipocytes.

Figure 4.8A shows that the addition of DH-DhL inhibited the differentiation of 3T3-L1 preadipocytes. The inhibition was dose-dependent and required a higher concentration of DH-DhL to produce a similar inhibitory effect as DhL. Thus, it required 10 times the DH-DhL concentration to achieve the same effect as DhL (Figure 4.8A). Furthermore, it was also found that 100 μ M of DH-DhL did not affect cell viability (control cells: $98 \pm 2\%$ of viability vs. 80 μ M DH-DhL treated cells: $97 \pm 2\%$ of viability). These results suggest that the reduction of the α -methylene- γ -lactone group of the DhL was not required to block the differentiation of 3T3-L1 preadipocytes.

To further investigate the role of these epimers of DH-DhL on the differentiation of 3T3-L1 preadipocytes, the two methyl epimers at C-11 produced during the *in vitro* reduction of DhL were separated by preparative HPLC, concentrated, confirmed by GC analysis and then their effect on adipogenesis was examined. For this, 3T3-L1 pre-adipocytes were incubated with 80 μ M of either (11S)DH-DhL or (11R)DH-DhL during the entire differentiation process. It was found that the addition of (11R)DH-DhL epimer, but not (11S)DH-DhL epimer, inhibited the differentiation of 3T3-L1 preadipocytes (Figure 4.8B). These

results suggest that (11R)DH-DhL epimer may be responsible in inhibiting adipocyte differentiation.

Several studies have shown that compounds that have the α -methylene- γ -lactone group can react with nucleophiles, especially cysteine sulfhydryl groups. Interestingly, studies indicate that cysteine residues in certain proteins, such as NF- κ B, may be direct targets of sesquiterpene lactones (Semenkovich, 1997). Thus, it is logical to speculate that proteins like Rin1 and Rab5 (Chapters 2 and 3), whose activities are required for adipogenesis, may be affected by DhL. Expression of Rab5 and Rin1, as well as Rab5 activity, showed a strong effect on adipogenesis: reducing lipid formation, decreasing the expression of key without affecting the major signaling pathways. To investigate the possible effect of DhL on Rin1 and Rab5 function, it was decided to study whether expression of Rin1 and Rab5 and Rab5 activity were affected by the addition of DhL. Interestingly, expression of Rin1 and Rab5 had shown a detrimental effect on adipogenesis, which is consistent with the inhibitory effect of the active form of Rab5 in adipogenesis (unpublished data Nicole Villaverde, 2009). To this end, 8 μ M DhL was added to cells and the level of expression of Rab5 and Rin1 were examined by Western blot analysis. Rab5 activity was determined by the formation of enlarged Rab5-positive endosomes in cells overexpressing Rab5:WT and the constitutively active mutant Rab5:Q79L (Barbieri et al., 1996). Overexpression of Rab5:WT promotes endosomes with an average perimeter of 1.2 $\pm$ 0.01 μ m, while the overexpression of the constitutively active mutant promotes endosomes with an average perimeter of 2.3 $\pm$ 0.012 μ m (Li and Qian, 2002). Figure 4.9A

and B shows that DhL did not affect either the expression or activity of Rab5, or the expression of Rin1 (Figure 4.10). Taken together, these observations reveal that DhL inhibited adipogenesis by a Rin1/Rab5 independent mechanism(s).

Figure 4.1. Dehydroleucodine inhibited adipogenesis of 3T3-L1 preadipocytes without reducing cell viability.

Dehydroleucodine inhibited adipogenesis of 3T3-L1 preadipocytes without reducing cell viability. **(A)** 3T3-L1 preadipocytes were differentiated into adipocytes in the absence or in the presence of various amounts of DhL (0.65 to 10 μ M) as described in Material and Methods. Results were represented as relative lipid contents. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to DMSO and only induction media-treated cells. **(B)** Cells were treated with 8 μ M DhL for either 24 hrs (day 1) or for 9 days (day 9). Cell viability was measured using the MTT assay as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments.

Figure 4.2. Dehydroleucodine blocked the formation of lipid droplet by induction media in 3T3-L1 cells.

Dehydroleucodine blocked the formation of lipid droplet by induction media in 3T3-L1 cells. Adipocyte differentiation was induced by treating confluent 3T3-L1 preadipocytes with induction media in the absence or presence of 8 μ M DhL. Morphological changes of 3T3-L1 preadipocytes were monitored by a microscope and photographed after 9 days from the onset of differentiation. **(A)** Vehicle only, **(B)** cells treated with induction media in the presence of DMSO, or **(C)** in the presence of DhL. **(D)** Nine days after induction of differentiation, cells were lysed for triglyceride and protein assays as described in Material and Methods. Vehicle only (line 1), cells treated with induction media alone (line 2), cells treated with induction media in the presence of DMSO (line 3), and cells treated with induction media in the presence of 8 μ M DhL (line 4). Bars=10 μ m. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to DMSO-treated cells and only induction media-treated cells.

Figure 4.3. Dehydroleucodine attenuated the expression of PPAR γ during 3T3-L1 preadipocyte differentiation.

Dehydroleucodine attenuated the expression of PPAR γ during 3T3-L1 preadipocyte differentiation. 3T3-L1 preadipocytes were induced to differentiate by induction media into adipocytes in the absence (inset: -DhL, control) or in the presence (inset: +DhL) of 8 μ M DhL. Total protein extracts were prepared at day 9 from each sample. The proteins were subset to 12% SDS-PAGE electrophoresis, blotted to a nitrocellulose membrane, and probed with antibodies specific to **(A)** PPAR γ , C-EBP α and tubulin, **(B)** P-Erk1/2, T-Erk1/2, **(C)** P-Akt1, T-Akt1, **(D)** P-AMPK α and T-AMPK α respectively. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to DMSO induction media-treated cells (control).

Figure 4.4. Dehydroleucodine selectively alters the expression of PPAR γ , C-EBP α and AMPK α .

3T3-L1 preadipocytes were induced to differentiate by induction media into adipocytes in the absence (insert: -DhL, control) or in the presence (insert: +DhL) of 8 μ M DhL. Total protein extracts were prepared at day 1, 3, 5, 7 and 9 from each sample. The proteins were subset to 12% SDS PAGE electrophoresis, blotted to a nitrocellulose membrane, and probed with anti-bodies specific to PPAR γ (A), C-EBP α (B), AMPK α (C), and tubulin. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to DMSO induction media-treated cells (control).

Figure 4.5. Dehydroleucodine blocked adipocyte differentiation in a time-dependent manner.

(A) 3T3-L1 preadipocyte cells were treated with 8 μ M DhL for the time indicated in the schematic representation of the experiment. **(B)** In each treatment (1–6), the accumulation of lipid droplets was measured by the incorporation of Oil Red O as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P < 0.05 and ** P < 0.01 by Student's *t*-test compared to DMSO induction media-treated cells (control).

Figure 4.6. Dehydroleucodine alters the expression of FAS.

3T3-L1 preadipocytes were induced to differentiate by induction media into adipocytes in the absence (insert: -DhL, control) or in the presence (insert: +DhL) of 8 μ M DhL. Total protein extracts were prepared at day 0 (no induction media), 1, 3, 5, 7 and 9 from each sample. The proteins were subset to 12% SDS PAGE electrophoresis, blotted to a nitrocellulose membrane, and probed with anti- FAS antibody. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments. * P <0.05 by Student's t-test compared to DMSO induction media-treated cells (control).

Figure 4.7. GC analysis of dehydroleucodine and 11,13-dihydrodehydroleucodine epimers.

(A) DhL isolated from *Artemisia douglasiana*; (B) mixture DH-DhL epimers as obtained by reduction of DhL, (#) denotes small amount of DhL after the reduction reaction; (C) DH-DhL epimer S, and, (D) DH-DhL epimer R after separation from the mixture. Chemical structures of DhL (compound 1), DH-DhL epimer 11S (compound 2) and DH-DhL epimer 11R (compound 3) (numbering according to classical nomenclature).

Figure 4.8. 11,13-Dihydro-dehydroleucodine inhibited 3T3-L1 preadipocyte differentiation.

(A) 3T3-L1 preadipocytes were differentiated into adipocytes in the absence or in the presence of either DHL or DH-DhL. Results were represented as relative lipid content. Data represent the mean $\pm$ S.E.M. of three independent experiments.

(B) 3T3-L1 preadipocyte cells were incubated with induction media supplemented with either DMSO, 80 μM DH-DhL epimer S or 80 μM DH-DhL epimer R and the incorporation of Oil Red O was measured by as described in Material and Methods. Results were represented as relative lipid content. Data represent the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.01$ by Student's t-test compared to DMSO-treated cells.

Figure 4.9. Effect of dehydroleucodine in Rab5 expression and activation.

(A) To study Rab5 expression, NIH3T3 cells overexpressing GFP-Rab5 constructs were exposed to 8μM DhL for 6hrs. Then, the cells were lysed and total protein extracts were subset to 12% SDS-PAGE electrophoresis, blotted to a nitrocellulose membrane, and probed with Rab5 and tubulin antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean±S.E.M. of three independent experiments. To study Rab5 activity NIH3T3 cells overexpressing GFP-Rab5 constructs were exposed to 8μM DhL for 6hrs. Then, the cells were fixed and the perimeter of Rab5-positive endosomes was measured, as described in Material and Methods, in cells overexpressing Rab5:WT **(B)** and Rab5:Q79L **(C)**.

Figure 4.10. Effect of dehydroleucodine in Rin1 expression.

NIH3T3 cells overexpressing Rin1 were exposed to 8 μ M DhL for 6hrs then lysed and total protein extracts were subset to 12% SDS-PAGE electrophoresis, blotted to a nitrocellulose membrane, and probed with Rin1 and tubulin antibodies. Relative levels of proteins were determined by densitometry as described in Material and Methods. Data represent the mean $\pm$ S.E.M. of three independent experiments.

V. DISCUSSION

Tyrosine kinase receptors (RTKs) are expressed in many cell types and regulate important biological processes such as cell growth and differentiation, among others (Brodeur et al., 2009; Shaham et al., 2008; Wieduwilt and Moasser, 2008). Even though RTKs are the focus of an extensive research area, its signaling and regulation have not been completely elucidated. Overactivation and/or malfunction of RTKs have been linked to hyperproliferative and other type of diseases. Therefore it is important to understand not only the pathway, but also the proteins involved in RTK signal transduction and trafficking. It is also critical to investigate the molecular mechanism by which these proteins are interconnected.

The scope of this dissertation is a detailed analysis of the function of Rin1, a cytosolic protein involved in RTK signaling and internalization. The aim of this study is to: 1) determine how key residues of Rin1 are responsible for the protein-protein interactions, 2) elucidate how these interactions impact biological processes, mainly cell differentiation, and 3) determine the role of small molecules on intracellular pathways leading to selective inhibitory effects. Misregulation of pathways involved in these biological processes may be a direct cause of certain diseases such as cancer, obesity and neurodegenerative diseases.

The present study shows that Rin1, through several domains, directly interacts with EGFR, IR, and NGFR upon ligand stimulation. Rin1-EGFR interaction was only observed when Rin1:WT or Rin1:N-terminal domain were

utilized, but not with the Rin1:C-terminal domain. Moreover, NGFR showed a similar type of interaction as the one observed for EGFR: through the SH2 domain. There was no interaction when the C-terminus of Rin1 was utilized. However, the IR-Rin1 interaction was not only through the SH2 domain, but also with the Vps9 (located at the C-terminal region, suggesting a novel interaction with the Vps9 domain and IR).

The crystal structure of SRC kinase shows the importance of a signature motif composed of Phe-Leu-Val-Arg-Glu-Ser (FLVRES) in SH2 domain function (Campbell and Jackson, 2003). Mutations in the arginine residue prevent almost every interaction with phosphotyrosines (Campbell and Jackson, 2003). In Rin1, residue arginine 94 was mutated to alanine creating Rin1:R94A mutant to investigate its effect in IR interaction and signaling. It can be concluded that even though this residue, does not affect the protein-protein interaction; it is critical for the proper function of the receptor tail.

Next, it was studied how this RTK-Rin1 interaction affected the internalization and signaling processes. Rin1 affects endocytosis and signal transduction via different proteins such as Rab5 and Ras. In the case of Rab5, Rin1 acts as a GEF, promoting the exchange from GDP to GTP (Han and Colicelli, 1995; Tall et al., 2001). As a consequence, the ligand-receptor complex internalization is accelerated, until the complex is separated in the late endosome and they both get delivered to the lysosome. The rate of endocytosis has a direct effect on receptor signaling: the faster the internalization, the shorter the signaling. This observation indicates that Rin1 not only regulates membrane

receptor signaling by activating Rab5 and accelerating the internalization process, but also by decreasing Ras signaling. Thus, when Rin1 is overexpressed in the cell upon ligand stimulation, Erk and Akt activities are decreased. Directed mutagenesis studies reveal that tyrosine 561 in the Vps9 domain may be one of the key amino acids for Rin1 function (Galvis et al., 2009). It has been shown that point mutation in this residue inhibit Rab5 activation (Galvis et al., 2009). In addition, it was found that tyrosine 561 also has an effect on the interaction of Rin1 with Ras (Galvis et al., 2009). Even though the Rin1:Y561F mutant does not have a direct role in the interaction, it reverses the inhibition of Erk and Akt by overexpression of Rin1:WT. Another residue that regulates Rin1 function is arginine 629 in the RA domain. It reduces the Ras-Rin1 interaction, which inhibits both, Erk and Akt activities. These data suggest a dual role of Rin1 domains: promoting Rab5 activation and decreasing Ras signaling upon ligand stimulation.

Phosphorylation of EGFR and IR also appears to be affected by the overexpression of Rin1, another possible cause of accelerating its internalization, decreasing the signal span. Similarly, when Rin2 and Rin3 are overexpressed, phosphorylation of the receptor decreases; there is an increase in Rab5 activation that may help to explain the effect of the Rin family. In contrast, the overexpression of the other two members of the Rin family (Rin2 and Rin3) seems to have a more cell-specific effect on Ras signaling. Rin2 promotes Erk activation upon EGF stimulation, while decreasing it upon insulin stimulation.

Consistent with this observation, Rin proteins show a distinctive tissue distribution (Gurkan et al., 2005).

Taken together, Rin proteins have a regulatory role in RTKs internalization and cell signaling. Rin1 shows a selective method of interaction with RTKs. This study describes a novel mechanism of interaction of Rin1 with IR, which is not observed with EGFR or NGFR. It also demonstrates that all domains are required for the regulatory role of Rin1 in RTK signaling.

Next, it was studied the interaction of Rin1 with membrane receptors, and its effect on Rab5 and Ras activation affected some biological processes such as, proliferation and cell differentiation. It has been shown that Rin1 affected cell proliferation as well as thymidine incorporation upon ligand stimulation (Hunker et al., 2006b). To determine the importance of Rin1 function in cell differentiation, it was decided to investigate two different and well-characterized cell differentiation processes: adipogenesis and neurogenesis.

In the case of adipogenesis, overexpression of Rin1 full-length inhibited significantly adipocyte differentiation by decreasing the expression of the key transcription factors PPAR γ and C/EBP α . Previous studies (unpublished data Nicole Villaverde, 2009) showed that Rab5 overexpression inhibits adipogenesis *in vitro* in 3T3-L1 preadipocytes. The inhibition by Rab5 may be, at least in part, because its activation promotes receptor degradation terminating its signal. Another explanation could be the role of Rab5 on the endosomal localization of EEA1 (Simonsen et al., 1998), as well as the nuclear localization of AAPL1 (Miaczynska et al., 2004). Inhibition *in vitro* of adipocyte formation by Rin1

overexpression confirms and also expands these data, since Rin1 is a Rab5 GEF. Thus, inactivation of Rab5 must be required for the normal process of adipogenesis. Actually, a decrease in adipocytes formation was observed when all members of the Rin family were overexpressed; Rin1 showed the greatest inhibitory effect.

Rab5 activation may not be the only factor explaining the inhibitory effect of Rin1 in preadipocyte differentiation. Each individual domain of Rin1 seems to have the same or similar inhibitory effect, indicating a high level of complexity of Rin1 function. Consistent with this observation, individual mutations in selective domains of Rin1 partially reversed the inhibitory effect of Rin1:WT. These data indicate that all domains of Rin1 and its respective interactions with RTKs, Rab5 and Ras are necessary for its role in adipogenesis.

In the case of neurogenesis, several studies have shown that Rab5 activity inhibits neurite growth, and it has been shown to be related to inactivate Rab5 through RabGap5 interaction (Liu et al., 2007).

Even though each individual RTK signaling pathway has been extensively studied, there is no complete understanding of how signal transduction networks integrate upon simultaneous stimulus (Volinsky and Kholodenko, 2013). Crosstalk between signal transduction pathways occur at different levels: receptors, adaptor proteins, GTPases, and transcription factors can be involved in more than one pathway at a time resulting in different outcomes (Pawson et al., 2001). Some RTKs activate the same signaling pathways with different biological outcomes. Upon NGF stimulation, PC12 cells will differentiate, but

upon EGF stimulation they will proliferate (Marshall, 1995). The key factor here seems to be the phosphorylation span of Erk. NGF-stimulated Erk lasts for hours, while the EGF-stimulated is just transiently activated (Heasley and Johnson, 1992). Rin1 overexpression in PC12 has no effect in neurite growth upon NGF stimulation. Thus, until now adipogenesis may be the only differentiation process affected by Rin1.

As indicated in Chapter 1, several small molecules have been implicated in different enzymatic activities, for example, RTKs inhibitors (Han et al., 1996). As described above, Rin1 and Rab5 have an effect in adipogenesis. In addition, other proteins (AMPK, C/EBP α and PPAR γ) have been shown to be required in different steps of the differentiation processes (Hwang et al., 1997).

The small molecule DhL showed a selective inhibitory effect in adipogenesis (Galvis et al., 2011). It attenuated dramatically the production of adipogenic transcriptional factors PPAR γ and C-EBP α during adipogenesis without altering the activation of Erk and Akt. In addition, it was also found that DH-DhL, a derivative of DhL with inactivated α -methylene- γ -lactone function, also inhibited the formation of adipocytes. However, it required ten times the DH-DhL concentration to achieve the same effect as DhL. Based on these observations, it is clear that in the adipocyte differentiation model, the α -methylene- γ -lactone moiety causes a significant decrease of 3T3-L1 preadipocyte differentiation at lower concentrations without altering cell viability. The molecular mechanism by which DhL and DH-DhL inhibit adipogenesis is not completely understood, but based on this work it can be concluded that it is

independent from the expression and activation of Erk, Akt, Rin1 and Rab5. It is important elucidate the direct target(s) of DhL because it can be utilized as a potential treatment for obesity and other related diseases.

FUTURE WORK

Further studies need to be carried out in order to understand the role of Rin1 in diseased cells at both, cellular and animal levels. First, it would be necessary to study the effect of the Rin1-RTK interaction, through the SH2 domain, in receptor degradation and signal attenuation. Second, study if the role of Rin1 through its interaction with Rab5 and Ras is cell/tissue-specific, by using other isoforms of the GTPases and examine the effect of these interactions in signal transduction and receptor trafficking. Third, to elucidate the molecular mechanisms used by Rin1 (and the small molecule DhL) to inhibit adipogenesis. All this knowledge may lead to a possible treatment for adipogenesis regulation and other diseases related to RTKs signaling and trafficking misregulation.

MATERIALS AND METHODS

Cloning.

Full length proteins and their domains (cDNAs) were cloned in the following vectors according to the technique used: pGEX-4T-1 (for the production of GST fusion proteins),t pB42AD and pLexA (for proteins used in the yeast two-hybrid system) and pMX-puro (for retrovirus production).

Point mutations were done using the QuickChange kit by Strategene following the manufacturer's protocol, except for the following mutations that were done by Genscript: Rin1:K642A/K662A/L675A and R702A. Oligonucleotide primers for cloning and mutagenesis were purchased from Sigma.

Construction of recombinant retroviruses and stable cell lines expressing protein constructs.

Rin1:WT, Rin1 mutants, Rin2:WT, Rin3:WT and Rab5:WT were sub-cloned into EcoRI restriction sites of the pMX-puro vector (Schlessinger, 2002; Zhu et al., 2001). To make stable cell lines expressing the constructs mentioned above, Plat-E packaging cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Han and Colicelli, 1995; Wang et al., 2002).

Rin1 depletion in 3T3-L1 cells.

3T3-L1 cells were transfected with lipofectamin and 100pmol RNAi against Rin1 either for one time (day 1) every 24hrs or every 48hrs during the differentiation process. At day 10, cells were either lysed and Western blotting analysis was

performed to confirm Rin1 depletion, or the cells were fixed for lipid content quantification.

Lysate preparation, SDS-PAGE and Western blotting.

To prepare whole cell lysates, cell monolayers were washed with phosphate buffer saline (PBS) and lysed in ice-cold lysis buffer (20mM Tris-HCL, 150mM NaCl, 1% Triton-X, 1mM PMSF, 1mM NaF, 1mM sodium orthovanadate). The lysates were clarified by centrifugation, and protein concentrations were determined by band intensities on SDS-PAGE gels stained with Coomassie Blue. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes, blocked and probed with the indicated antibodies. To determine relative protein amounts, three representative exposures for each sample were quantitated by densitometry analysis.

GST-fused protein purification.

pGEX-4T1 constructs (Rin1 full-length, domains and mutants) expressed in BL21 (DE3) RIPL Escherichia coli were induced with 0.25mM IPTG for three hours. Bacterial cells were resuspended in extraction buffer (50mM Tris-HCl, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, 1mM PMSF, 1mg/ml lysozyme, 10ug/ml DNase, 2ug/ml leupeptin, 2µg/ml aprotinin) and lysed by sonication and incubation with 1% Triton X-100. Protein concentrations were determined by band intensities on SDS-PAGE gels stain with Coomassie Blue.

Pull-down assay.

For *in vitro* studies, fusion proteins were expressed and purified as in the protein purification section. For the GST-Pull-down ten µg of immobilized GST alone and

GST-fused protein to glutathione sepharose beads were blocked with 0.1% BSA in extraction buffer and then were incubated with different amounts of cell lysate for 1h at 4°C in binding buffer. After incubation, the beads were washed three times by centrifugation and bound proteins were eluted by the addition of SDS sample buffer. Solubilized proteins were separated by SDS-PAGE, and analyzed by Western blotting using the appropriate antibody.

Rab5 activation assay.

NR6-EGFR and NIH3T3-IR were stimulated as explained in the section of “Tyrosine kinase receptor-dependent signaling” below. The activation was done using the Rab5 activation assay kit following the manufacturer’s protocol (New East Biosciences). Briefly, total cell lysates were incubated with anti-Rab5-GTP mouse monoclonal antibody. Then, protein A/G agarose was used to pull down the bound Rab5-GTP and it was detected by Western blotting using rabbit anti-Rab5 antibody.

Ras activation assay.

NR6 cells expressing GFP and Rin1:WT were lysed using lysis buffer supplemented with 1 mM PMSF. Lysates (1 ml) were then incubated with 100µl glutathione beads containing ~10 µg of GST-RAF at 4°C while rocking for 1hr. After incubation, the beads were washed three times using the lysis buffer. The pull-downs were subjected to SDS-PAGE and analyzed by Western Blotting using an anti-pan Ras antibody. Relative Units (% of maximum) of proteins were determined by densitometry using the ratio of active Ras to total-Ras.

Insulin receptor tail phosphorylation.

Six μ l of pure recombinant Insulin receptor tail were diluted in a buffer containing 10mM $MgCl_2$, 240mM NaCl, 0.05% Triton-X 100, 50mM Tris HCL and 20 mM ATP. The reaction was left 30 minutes at room temperature prior to the binding to the GST beads explained above.

Immunoprecipitation assay.

All immunoprecipitations were performed on cytosol prepared from cells in ice-cold buffer containing several phosphatase and protein inhibitors as described above. After clarification, protein concentrations were measured and samples were immunoprecipitated by incubation with the appropriate antibodies followed by immobilization on Protein G-Sepharose beads (Amersham Pharmacia Biotech). The beads were washed three times with buffer and the bound proteins were resuspended in sample buffer and analyzed by SDS-PAGE and Western blot with the indicated antibodies.

Yeast Two-Hybrid assay.

Two-hybrid LexA DNA binding domain (LexA-BD) and B42 transcription activation domain (B42AD) fusion constructs were prepared by ligating cDNAs for different proteins or protein fragments into the pLexA (His) and pB42AD (Trp) plasmids, respectively (Clontech). Mating and transformation of Yeast-EGY48 cells were done as described in the MATCHMAKER two-hybrid manual (Clontech). The Rin1 constructs were expressed as pB42AD fusion constructs whereas Rab5:S34N, HRas:V12, insulin receptor and insulin receptor: kinase dead mutants were expressed as pLexA fusion constructs. Yeast-EGY48 co-

transformants were plated on medium without His (H), Trp (W), Leu (L) or Ura (U) (-HWUL) to detect LacZ reporter gene activation due to interaction of constructs and on medium without His, Trp, or Ura (-HWU) as a control for loading and growth on galactose containing medium. All Rin1, Rab5, Ras and insulin receptor constructs were detected by Western blot analysis. For liquid β -galactosidase (β -gal) assays, yeast was grown on appropriate selective media and assayed as described in the yeast β -gal assay manual (Pierce). Results were expressed in Units: one unit of β -gal was defined as $1000 \times \text{OD}_{420} / [\text{time} \times \text{vol} \times \text{OD}_{660}]$.

Tyrosine kinase receptor-depending signaling.

NR6-EGFR/ NIH3T3-IR/ PC12 cell monolayers were infected with pMX viruses (alone or Rin1 proteins) and were serum starved for 3hrs prior to activation with 100 ng/ μ l EGF, 100nM insulin or 100nM NGF respectively, for 5 minutes. Cells were washed with PBS and lysed (20mM Tris-HCL, 150mM NaCl, 1% Triton-X, 1mM PMSF, 1mM NaF, 1mM sodium orthovanadate). Cell proteins were separated by SDS-PAGE, and analyzed by Western blotting using the appropriate antibody for different signaling proteins (i.e., phospho and total-Erk, Akt, p38 and Junk). Relative levels of the proteins were determined by densitometry.

3T3-L1 preadipocyte differentiation.

3T3-L1 cells (ATCC) were grown to confluence in DMEM high glucose supplemented with 10% fetal bovine serum, 1% penicillin/ streptomycin and 1% L-glutamine (growth media) in a humidified atmosphere of 5% CO₂ at 37°C.

Culture was fed every 48hrs, both for cell growth and differentiation. To trigger differentiation cells were exposed to differentiation medium (growth media supplemented with 670nM insulin, 65nM dexamethasone and 0.5mM 3-isobutyl-1-methylxanthine [IBMX]) for the first two days, then fed with post-differentiation media (DMEM high glucose supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine and 670nM insulin) for the next seven days. DhL treated cells were exposed to the compound the entire process with different concentrations, unless otherwise indicated.

High-performance liquid chromatography analysis.

The high-performance liquid chromatography (HPLC) equipment consisted of a SpectraSystem SMC1000 solvent delivery system, vacuum membrane degasser, P4000 gradient pumps and AS3000 autosampler (Thermo Electro Corporation). Column effluent was monitored at 254 nm with Spectra System UV6000LP variable wavelength PDA detector and ChromQuest 4.1 software. DhL, (11S)DH-DhL and (11R)DH-DhL were separated using a C18 YMC column (A-302, 150×4.3 mm i.d., S-5 µm, 12 nm; Waters) and the following solvents: A. acetonitrile; and B. 0.1% TFA in water. System 1: linear gradient 10% to 100% A in 120 minutes; flow rate 1 ml/min. Preparative HPLC was performed in the above equipment with a XTerra Prep MS C18 OBD column, 15 µm, 19×50 mm (Waters) and the solvent system 25% acetonitrile– 75% 0.1% TFA in water (isocratic); flow rate 3 ml/min.

Gas chromatography (GC)/flame ionization detector (FID) and GC/ mass spectrometry (MS) analysis of DhL derivatives.

GC/FID analyses were performed on a Trace GC Ultra apparatus (Thermo Electro Corporation) equipped with a flame ionization detector. The output was recorded using a ChromQuest version 4.1 data system. A DB-5MS capillary column (0.25 mm i.d. × 30 m; film thickness 0.25 µm; J & W Scientific) was employed. The temperature was programmed 105 to 240°C at 3°C/min (linear increase) and then the temperature was held at 240°C for 10 minutes. The injector temperature was 250°C with a split ratio of 1/20. The detector temperature was 270°C. Helium was used as gas carrier at 1 ml/min. DHL, (11S)DH-DHL and (11R)DH-DhL were dissolved in ethyl acetate and 2µl of the solution was injected. GC/MS determinations were carried out in a Hewlett-Packard model 6890 instrument coupled to a Q-Mass 910 quadrupole selective detector at 70 eV and equipped with a DB-5MS capillary column. Temperature program and other conditions were as indicated above.

DhL derivatives: compound identification.

Dehydroleucodine {(1S,6S,2R)-9,13-dimethyl-5-methylene-3oxatri- cyclo
[8.3.0.0b2,6N] trideca-9,12-diene-4,11-dione, IUPAC nomenclature}. DhL
(compound 1): HPLC (System 1), Rt 27.74 min; GC/MS, Rt 22.72 min; UV/PDA
λ_{max} 256 nm; MS m/z (rel. int.), 244 (100) M⁺, 173 (18.2), 145 (19.8), 129 (18.9),
115 (18.5), 105 (18.3), 91 (62.9), 79 (17.7), 77 (28.4), 65 (21.6), 53 (30.8).
(1S,2S,5S,6S)-5,9,13-trimethyl-3-oxatricyclo[8.3.0.0b2,6N]trideca-9, 12-diene-
4,11-dione;11,13-dihydro-dehydroleucodine (IUPAC nomenclature), (11S)DH-

DhL (compound 2): HPLC (System 1), Rt 28.16 min; GC/MS, Rt 21.87; UV/PDA λ_{max} 257 nm; MS, m/z (rel. int.): 246 (100) M⁺, 217 (26.7), 173 (37.6), 172 (31.2), 145 (26.2), 105 (23.2), 91 (60.7), 77 (26.2), 55 (24.3). (1S,2S,6S,5R)-5,9,13-trimethyl-3-oxatricyclo[8.3.0.0b2,6N]trideca-9, 12-diene-4,11-dione, 11,13-dihydro-dehydroleucodine (IUPAC nomenclature), (11R)DH-DhL (compound 3): HPLC (System 1), Rt 26.23 min; GC/MS, Rt 22.84; UV/PDA λ_{max} 257 nm; MS, m/z (rel. int.): 246 (100) M⁺, 217 (31.5), 173 (35.5), 172 (33.1), 145 (27.5), 105 (26.1), 91 (67.1), 77 (29.0), 55 (25.8).

Effect of DhL in Rin and Rab5 expression and activation.

NIH3T3 cells overexpressing GFP-Rab5 or Rin1 were exposed to 8 μ M DhL for 6hrs. For protein expression, the cells were lysed and total protein extracts were subset to 12% SDS-PAGE electrophoresis, blotted to a nitrocellulose membrane, and probed with Rab5 or Rin1, and tubulin antibodies. For Rab5 activity, the cells were fixed with 2% paraformaldehyde for 20 minutes, and the diameter and perimeter of Rab5-positive endosomes were measured using the ImageJ64 software.

Adipogenesis quantification.

On the tenth day of differentiation cells were fixed with 10% formalin in PBS 1hr at 4°C. Oil Red O (Allied Chemical) stock solution (0.6g in 100ml of isopropanol) was diluted with 0.6 parts of water, filtered and added to the fixed cells for 15 minutes at room temperature. Cells were washed with water and analyzed in an inverted microscope (photos of lipid droplets were taken). To quantify the lipid droplets, Oil Red O was eluted with 100% isopropanol for 10 minutes at 37°C,

collected, and its optical density was measured at 540 nm. Lipid quantification was verified by studying the expression levels of adipogenic differentiation markers, such as of AMPK, FAS, PPAR γ , and C-EBP α by Western blotting as explained above.

Neurogenesis.

PC12 cells were grown to confluence in DMEM high glucose supplemented with 5% fetal bovine serum, 10% horse serum, 1% penicillin/streptomycin and 1% L-glutamine (growth media) in a humidified atmosphere of 5% CO₂ at 37°C. Culture was fed every 48hrs, both for cell growth and differentiation. To trigger neurogenesis cells were exposed to differentiation medium (DMEM, 1% horse serum, 1% penicillin/streptomycin and 100ng/ml NGF) during the 10 days treatment. Neurite outgrowth was monitored daily and cell processes longer than one cell body in diameter was counted as neurites.

Statistical analysis.

All experiments presented were repeated a minimum of 3 times. The data represent the mean $\pm$ SEM. Student's t test was performed to calculate statistical significance.

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APPENDICES

Appendix 1

Primers used to do the point mutations in Rin1.

1) Rin1: W69E

5' - CTCACCCGGCCCGTGGAGCTGCAGCTGCAAGCCAAC - 3'

2) Rin1: A76E

5' - CTGCAGCTGCAAGCCAACGAAGCGGCCGCACTGCAC - 3'

3) Rin1: R94A

5' - CCGGGGACGTTCTCTCGTGGCGAAATCTAACACCCGCCAG - 3'

4) Rin1:Y561F

5' - GAGCTGCTGCTGGCGGCCGAGTTCATGTCTGGAGCTGCTGGAGCCCAG
- 3'

5) Rin1:T580A

5' - GAGGGTGGCTACTACCTGGCCAGCCTCTCTGCCAGCCTG - 3'

6) Rin1:R629A

5' - CACCTCCTCGCAGTAGCCTATC - 3'

7) Rin1: H120L

5' - CCCTCCTTCGTCTCCAGCCTCTACATCCTGGAGAGCCCTGGC - 3'

8) Rin1: Y121F

5' - CCCTCCTTCGTCTCCAGCCACTTCATCCTGGAGAGCCCTGGC - 3'

9) Rin1: Y148F

5' - CCCTCCTTCGTCTCCAGCCACTTCATCCTGGAGAGCCCTGGC - 3'

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