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Modulating Adipogenesis: Key Role of Ras-related Protein Rab5 and its Effectors

Yongjun Huang
hyong001@fiu.edu

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

MODULATING ADIPOGENESIS: KEY ROLE OF RAS-RELATED PROTEIN
RAB5 AND ITS EFFECTORS

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY

by

Yongjun Huang

2018

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Yongjun Huang, and entitled Modulating Adipogenesis: Key Role of Ras-Related Protein Rab5 and Its Effectors, 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.

Fernando Noriega

Lou W. Kim

Xiaotang Wang

Alejandro Barbieri, Major Professor

Date of Defense: June 25, 2018

The dissertation of Yongjun Huang is approved.

Dean Michael R. Heithaus
College of Arts, Sciences and Education

Andres G. Gil
Vice President for Research and Economic Development
And Dean of University Graduate School

Florida International University, 2018

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ABSTRACT OF THE DISSERTATION
MODULATING ADIPOGENESIS: KEY ROLE OF RAS-RELATED PROTEIN
RAB5 AND ITS EFFECTORS

by

Yongjun Huang

Florida International University, 2018

Miami, Florida

Professor Alejandro Barbieri, Major Advisor

The formation of adipocytes is a complicated process in which insulin and IGF-1 signaling pathways and numerous transcription factors control the conversion of precursor cells to mature fat cells. The Rab5 protein acts as a rate-limiting protein during receptor-mediated endocytosis by switching between a GDP-bound inactive form and a GTP-bound active form. The inactivation and activation of Rab5 are regulated by several Rab5 GTPase activating proteins (GAPs) and Rab5 guanine nucleotide exchange factors (GEFs), respectively.

This dissertation demonstrated that the activity of the small GTPase Rab5 and its regulators are essential for the differentiation of 3T3-L1 pre-adipocytes. Specifically, it showed that Rab5 activation is detrimental to the differentiation process. The overexpression of a dominant-negative Rab5:S34N mutant, but not an active counterpart (Rab5:Q79L), stimulated the differentiation of 3T3-L1 pre-adipocytes. Consequently, the expression of Rab5:S34N increased the expression of two adipogenic-specific transcriptional factors, PPAR γ and C/EBP α . siRNA-mediated depletion of Rab5 inhibited the differentiation of 3T3-L1 pre-adipocytes,

providing further evidence for the requirement of Rab5 in the process of adipogenesis.

A dramatic decrease of the Rab5-GTP level is also observed during the differentiation of 3T3-L1 pre-adipocytes. Consistent with these observations, I found that the expression of Rab5 GEFs (i.e., RIN1, Rabex-5, and RAP6), which increased the GTP-bound form of Rab5, blocked the differentiation process. In contrast, the expression of Rab5 GAPs (i.e., RN-tre and RabGAP-5), which decreased the GTP-bound form of Rab5, stimulated differentiation of 3T3-L1 pre-adipocytes.

I also found a novel interaction between the VPS9 domain of the Rab5 GEFs and the activated insulin receptor. This interaction is specific since the VPS9 domain did not interact with the catalytic inactive mutant of the insulin receptor and the Rab5 GAPs (no VPS9 domain) did not bind to the activated insulin receptor.

The data point out that a reduction on the GTP-bound form of Rab5 is required for the rapid differentiation of 3T3-L1 pre-adipocytes, identifying Rab5 inactivation as an important contributor of adipogenesis. Also, these observations suggest a novel cellular mechanism of Rab5 activity in the adipogenesis process in connection with the insulin receptor, the Rab5 GAPs, and the Rab5 GEFs.

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ABBREVIATIONS

ACBP: acyl-CoA-binding protein

ACC: acetyl-CoA carboxylase

ACS: acyl-CoA synthetase

AGPAT: 1-acylglycerol-3-phosphate acyltransferase

Akt: Protein kinase B

APS: adapter protein containing a PH and SH2 domain

ATGL: adipose triglyceride lipase

BAT: brown adipose tissue

BDNF: brain-derived neurotrophic factor

BHK: body hamster kidney

BMI: body-mass index

BMP: bone morphogenetic protein

BRFSS: Behavioral Risk Factor Surveillance System

C/EBP: CCAAT/enhancer-binding protein

cAMP: cyclic adenosine monophosphate

CC: coiled-coil

CCP: clathrin-coated pit

CCT: CTP:phosphocholine cytidylyltransferase

CDK2: Cyclin-dependent kinase 2

CHC: clathrin heavy chain

CHRD1: chordin and chordin-like-1

Cide: cell death-inducing DFF45-like effector

CLC: clathrin light chain

CME: clathrin-mediated endocytosis

DAG: diacylglycerol

DGAT: diacylglycerol acyltransferase

EEA1: early endosome antigen 1

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ER: endoplasmic reticulum

Erk: extracellular signal regulated kinase

FA-CoA: fatty acyl-CoA

FABP4: fatty acid binding protein 4

FAS: fatty acid synthase

FAT: fatty acid transporter

FATP: fatty acid transport protein

FDA: Food and Drug Administration

FGF: fibroblast growth factor

G3P: glycerol 3-phosphate

GAP: GTPase activating protein

GAPex-5: GTPase activating protein and exchange factor of Rab5

GDF: GDI displacement factor

GDI: guanine nucleotide dissociation inhibitor

GDP: guanosine di phosphate

GEF: guanine nucleotide exchange factor

GFP: green fluorescent protein

GLUT4: glucose transporter type 4

GPAT: glycerol 3-phosphate acyltransferase

Grb2: growth factor receptor bound protein 2

GREB: cAMP response element-binding protein

GST: glutathione-S-transferase

GSV: GLUT4 storage vesicle

GTP: guanosine triphosphate

HB: helical bundle

HGF-1: hepatocyte growth factor 1

HSL: hormone-sensitive lipase

IGF-1R: insulin-like growth factor receptor 1

IGF: insulin-like growth factor

IL-1: interleukin 1

IP₃: inositol 1,4,5-trisphosphate

IR: insulin receptor

IRS: insulin receptor substrate

JNK: Jun N-terminal kinase

LD: lipid droplet

LDL: low density lipoprotein

MAPK: mitogen-activated protein kinases

MCE: mitotic clonal expansion

MEK: MAPK/Erk kinase

MOG: myelin oligodendrocyte glycoprotein

MSC: mesenchymal stem cell

mTORC1: mammalian target of rapamycin complex 1

Myf5: myogenic factor 5

NGF: nerve growth factor

NHANES: National Health and Nutrition Examination Survey

PAP: phosphatidate phosphohydrolase

PI: phosphoinositide

PI3K: phosphoinositide 3 kinase

PI3P: phosphatidylinositol 3-phosphate

PIP2: phosphatidyl inositol-4,5-bisphosphate

PIP3: phosphatidyl inositol-3,4,5-triphosphate

PKA: protein kinase A

PLC- γ 1: phosphoinositide phospholipase C

Plin: perilipin

PMSF: phenylmethane sulfonyl fluoride or phenylmethylsulfonyl fluoride

PPAR γ : peroxisome proliferator-activated receptor

PR: proline rich

PTB: phosphor-tyrosine-binding

RA: Ras association

Rab: Ras-related GTP-binding protein

Rab5: Ras-related protein 5

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

Raf: Rapidly Accelerated Fibrosarcoma

RAP6: Rab5-activating protein 6

Ras: rat sarcoma

Rb: retinoblastoma

RGC: RAL–GAP complex

RIN1: Ras interference 1

RME-6: receptor-mediated endocytosis protein 6

RME: receptor-mediated endocytosis

RN-tre: related to the N terminus of tre oncogene

ROS: reactive oxygen species

RTK: receptor-tyrosine kinases

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SH2: SRC homology 2

SNS: sympathetic nervous system

SOS: son of sevenless

Src: sarcoma

SVF: stromal vascular fraction

TAG: triacylglycerols

TGF- α/β : transforming growth factor alpha/beta

TKD: tyrosine kinase domain

TNF- α : tumor necrosis factor alpha

TrkA/B: tropomyosin receptor kinase A/B

UCP1: uncoupling protein 1

VEGF: vascular endothelial growth factor

VPS9: vacuolar protein sorting associated protein 9

WAT: white adipose tissue

WT: wild-type

β 3-AR: β 3-adrenergic receptor

CHAPTER I

I. Introduction

Adipose tissue includes a diversity of cell types, including endothelial cells, blood cells, fibroblasts, pre-adipocytes, and macrophages, with mature adipocytes as the main cell type.

Adipogenesis is an insulin receptor-driven signaling cascade that promote pre-adipocytes to differentiate into mature (brown and white) adipocytes through the participation of some key transcription factors (Lowe *et al.*, 2011). Specifically, adipogenesis is a programmed and highly-regulated process that requires factors like peroxisome proliferator-activated receptor γ (PPAR γ) and other transcription factor families, including CCAAT/enhancer-binding proteins (C/EBPs), signal transducers and activators of transcription (STATs), and Kruppel-like factor (KLF) proteins (Ali *et al.*, 2013).

On the other hand, the intracellular membrane trafficking of the insulin receptor is an extremely complex process during which the receptor encounters its adaptor proteins as well as a variety of enzymes. One of them, the small GTPase Rab5, acts as a rate-limiting protein and governs the early stage of insulin receptor-mediated endocytosis. How this cellular process is regulated by the small GTPase Rab5 and its effectors during adipogenesis is not clearly understood. The specific roles of these intracellular factors, including the transcription factors, Rab5, and Rab5 regulators, their targets and the corresponding signaling events will be described. Thus, the working hypotheses and specific aims are proposed and

discussed on the basis of the current knowledge and the information provided in this chapter.

I.1 Adipogenesis: the mystery of obesity

Along with the development of the society and the more comfortable and convenient accessibility of food source, obesity, as one of the most severe global health problems, receives attention all around the world. Meanwhile, adipogenesis has been extensively studied in the past four decades because of the increase in the number of obese people.

I.1.1 Obesity: a global health problem

I.1.1.1 Obesity in history and modern life: from a symbol of beauty to the threaten of health

Obesity is a medical condition in which excessive fat is formed and accumulated in the body with a body-mass index (BMI) $\geq 30 \text{ kg/m}^2$ (Haslam & James, 2005). Rather than be treated only as a metabolic disorder, obesity nowadays has been recognized as a multifactorial disease. Type 2 diabetes, high blood pressure, osteoarthritis, stroke, congenital disabilities, and menstrual disorders are related to the increase of body weight (Haslam & James, 2005). Besides, animal and human data show that high glucose/fat food consumption sabotage the appetite control by impairing hippocampal-dependent memories, which suggests obesity is a threat to the central nervous system (Morris *et al.*, 2015). What is more, the comorbidities not only reduce life expectancy and increase the economic burden of the society, but also are associated with

increased incidence and mortality of multiple types of cancers, including liver, colon, bladder, endometrium, and breast cancer (Ezzati *et al.*, 2004; Giovannucci *et al.*, 2010)

Obesity is derived from the Latin word *obesitas*. The ancient Greeks and Egyptians were the first ones on record to recognize obesity as a medical disorder (Heitmann *et al.*, 2012). In the 6th century B.C., an Indian surgeon Sushruta related obesity with diabetes and heart disorders (Dwivedi & Dwivedi, 2007). During the Middle Ages and the Renaissance (~1300 A.D. to 1700 A.D.), obesity was a sign of wealth and prosperity and the symbol of beauty, not only in Europe but also in Asian (Bloomgarden, 2003). The values towards beauty can be represented by the artistic works at that time, such as the Portrait of Alessandro del Borro, a Tuscan nobleman and soldier, who fought in the wars against Ottoman Empire, was painted as a confident and over obese man; the Madonna with the Christ Child and St. John the Baptist, in which Madonna and Jesus were represented as overtly obese woman and child; and the Venus at a Mirror and the Woman at the well, in which women were drawn as overweight (Ferrucci *et al.*, 2010). During the 19th century, both height and weight of human bodies increased in the developed world, which was believed to be associated with the efficient and sufficient access to food and accumulated society wealth as a result of the Industrial Revolution (Caballero, 2007). However, it happened quite recently (around the 1980s) that obesity attracted the sight of the public and started being extensively studied by researchers, and it was only until the 20th century that obesity was recognized to associate with mortality increase (Eknoyan, 2006).

Since the beginning of the 20th century, public's view towards ideal body weight and the relationship between obesity and wealth changed dramatically. The change is supported by the data that the height of pageant winners of Miss American increased less than 2%, but the trend of BMI decreased from 22 to 17.5, which even belongs to the range of undernutrition defined by the World Health Organization (WHO) (Rubinstein & Caballero, 2000).

Nowadays, increasing evidence demonstrates that obesity is becoming a global disease. According to the National Health and Nutrition Examination Survey (NHANES), in the U. S., during 1999-2002, 86.3% and 84.2% of men and women who were diagnosed as diabetes were obese or overweight with 53% and 58% of them being obese, respectively (Report, 2004). Studies also found that nearly 29% of the world population was overweight or obese in 2013, and nearly two out of three of the obese population lived in developing countries (Mckay, 2014). What is worse, the data of Behavioral Risk Factor Surveillance System (BRFSS) from 1990 to 2008, show that obesity prevalence increased by 33% and severe obesity prevalence increased by 130%. Researchers predicted that by the year 2030, 42% of the population will be obese with a severe obesity ($BMI \geq 40 \text{ kg/m}^2$) prevalence of 11% (Finkelstein *et al.*, 2011).

I.1.1.2 Adipose tissue: not only a fat storehouse

Obesity, as the name implies, always happen with the symptom of a gain of body weight. The gain of body weight, however, mostly attributes to the increase in the mass of adipose tissue.

Adipose tissue is loose connective tissue containing adipocytes, which are also the dominant component of adipose tissue; stromal vascular fraction (SVF), which includes adipocyte precursors, mesenchymal stem cells (MSCs), and endothelial precursor cells; and immune cells such as adipose tissue macrophages (Riordan *et al.*, 2009). The MSCs resemble fibroblasts morphologically and have the capacity to differentiate into adipocytes, chondrocytes, myocytes and osteocytes under proper stimulation (Zuk *et al.*, 2001). The endothelial precursor cells are capable of stimulating angiogenesis either directly or indirectly by secreting insulin-like growth factor 1 (IGF-1), hepatocyte growth factor 1 (HGF-1), and vascular endothelial growth factor (VEGF) (Rehman *et al.*, 2004; Sumi *et al.*, 2007). The adipose tissue macrophages are responsible for the production of the majority of tumor necrosis factor alpha (TNF- α), which affects glucose homeostasis in adipocytes, promotes lipolysis and inhibit lipogenesis, and has been shown to relate with insulin resistance and type 2 diabetes (Cawthorn & Sethi, 2008). Three types of adipocyte precursors, preadipocytes, the CD34⁺/CD31⁻ cell population, and the human multipotent adipose tissue-derived stem cells, were found in the SVF of human adipose tissue (Symonds, 2012). These adipocyte precursors, which have the capacity to differentiate into adipocytes, resident in adipose tissue during adult life, undergo self-renewal to produce adipocytes, and allow the adipose tissue to expand as much as 15-fold as a result of a constant excess of fat intake (Symonds, 2012).

Researchers mainly focused on the regulation of lipolysis and lipogenesis since adipose tissue was only considered to be an energy storage organ in the

past. They started realizing the importance and complexity of adipose tissue in the recent 20~25 years when adipose tissue was widely and extensively studied because of the increasing prevalence of obesity. Adipose tissue is crucial and multifunctional in the body. It is 1) the primary energy storage site, where excessive fat that surpasses the requirement of body will be stored; 2) the insulating layer, which reduces heat loss and maintains the body temperature; 3) the protect and support structure of some organs; 4) an endocrine organ that secretes numerous endocrine mediators, including hormones (adipokines), cytokines, and lipids; 5) an immune organ that helps in the removal of debris and apoptotic cells, and the maintenance of tissue homeostasis (Mraz & Haluzik, 2014; Vegiopoulos *et al.*, 2017).

However, the functions of adipose tissue have been found to be different and, to some extent, harmful to obese individuals. Adipose tissue changes histologically, functionally, and metabolically in the obese microenvironment. In obese patients, most adipose depots visibly expand in size, adipocytes increase in numbers and sizes, while the proportions of other types of cells also changed. Specifically, the expanded volume of adipose tissue and increased adipocytes cell number are principally the result of the reduced removal rate of triacylglycerol (TAG), the main component of fat in adipocytes (Arner *et al.*, 2011). Additionally, excessive accumulation of fat leads to an increase of number and activity of some immune cells, such as macrophages, but a decrease of some others (Cildir *et al.*, 2013). The elevated number of macrophages increases the secretion of TNF- α in both adipose tissue and the circulatory system, which is related to insulin resistant

in vitro (Plomgaard *et al.*, 2005). Besides, adipose tissue of obese patients has relatively reduced blood vessels and neural structure, which form a hypoxic microenvironment in adipose tissue, and are thought to be responsible for insulin resistant of obese patients (Coppack, 2005). In response to the hypoxia stress, adipocytes activate the p38 mitogen-activated protein kinases (p38 MAPKs) and the Jun N-terminal kinases (JNKs), which correlate with glucose uptake and insulin sensitivity (M. Bluher, 2013). The dysregulated secretion of TNF- α , hypoxia microenvironment, and the stress-induced changes in signaling pathways attribute to an obesity-induced specific type of inflammation named chronic low-grade sterile inflammation. This type of inflammation is characterized by the increase of immune factors in the circulatory system and the absence of clinical signs of inflammation. It is also believed to play a crucial role in the development of insulin resistance in obese individuals (Medzhitov, 2008; Oliver *et al.*, 2010). Overall, adipose tissue is crucial for energy control and the homeostasis of the human body. Its dysfunction in obese patients is the leading cause of the obesity-related diseases.

I.1.1.3 White, brown, and beige: different colors with different functions?

The two functionally distinct types of adipose tissue found in mammals are classified as the white adipose tissue (WAT) and the brown adipose tissue (BAT), where the white adipocytes and the brown adipocytes cluster, respectively. It is generally believed that under specific situations, the white adipocytes undergo a

“browning” process and gain the features of brown adipocytes. These white-original brown adipocytes are named beige, or brite adipocytes. However, evidence also shows that the beige cells could be a distinct type of adipocytes which arise from unique precursor cells that are different from the white or brown (J Wu *et al.*, 2012). The introduction of this dissertation will only focus on discussion of the universally brief of beige origin.

The WAT, taking up the majority of the adipose tissue, could be found both subcutaneously and viscerally, and is one of the most abundant tissues in the human body. The abnormal expansion/hypertrophy of WAT is the dominant factor to contribute to the weight gain and dysfunction of adipose tissue during obesity progression because of the fact that the WAT is poorly vascularized with relatively rare neural structures (Vazquez-Vela *et al.*, 2008). White adipocytes, the main component of WAT, manage the primary functions of WAT, such as rapid accumulation and release of fatty acids, lipogenesis, lipid storage, and regulation of insulin sensitivity (Symonds, 2012). Adipocytes arise from the mesoderm during fetal development (Enerback, 2009). The development of white adipocytes, for a long time, is believed to derive from a myogenic factor 5 (Myf5)-negative lineage (Timmons *et al.*, 2007). However, developmental studies challenged the view by providing evidence of the existence of Myf5-positive precursors in WAT (Figure 1). The mature white adipocytes are round-shaped cells variable in size from 25µm to 200µm. They contain a single giant lipid droplet in the cytoplasm and a flattened, peripherally located nucleus (Table 1). The content and function of mitochondria are one of the main differences between the white and brown adipocytes.

Mitochondria in white adipocytes are, like in other tissues, the main source of ATP, thin and spindle in shape, and though variable, still relatively few in numbers (Table 1). The dysfunction of mitochondria could cause the imbalance between production and decomposition of reactive oxygen species (ROS), which is thought to play a role in the metabolic dysregulation of obesity (Cedikova *et al.*, 2016).

The BAT was thought to only exist in infants and small animals to defend cold temperature and maintain body temperature in the past (Rothwell & Stock, 1979). But it has been found to locate in supraclavicular, paravertebral, and neck regions and been suggested to play a potential role in the treatment of obesity in the past three decades. As the name of BAT implies, the brown adipocytes comprise the main portion of it. The brown adipocytes, derive from the Myf5-positive cell lineage, are relatively smaller (size from 15 μ m to 60 μ m) polygonal cells with multiple scattered lipid droplets in the cytoplasm and a round, eccentrically, yet not peripherally, located nucleus (Table 1). Mitochondria in brown adipocytes are bigger and contain more cristae than the ones in white adipocytes. These mitochondria have unique elliptical shape, exist in the cytoplasm with enormous numbers, and exclusively express a transmembrane protein, uncoupling protein 1 (UCP1) (Figure 1). Together with high vascularization, the cytochromes of numerous mitochondria give the BAT the visible brown color (Table 1). Unlike the WAT whose regulation is principally controlled by insulin, the BAT is primarily innervated by the sympathetic nervous system (SNS), which responses to energy defect or increased energy expenditure, via activating of numerous-expressed β 3-adrenergic receptors (β 3-AR) on the membrane of

mature brown adipocytes (Table 1). The activation of $\beta 3$ -AR enhances the expression of UCP1 and hormone-sensitive lipase (HSL), and eventually promotes lipolysis and thermogenesis (Geerling *et al.*, 2014).

Figure 1. Origins and characteristics of white and brown adipocytes.

The white adipocytes are predominantly derived from $Myf5^-$ cells while the brown adipocytes are derived from $Myf5^+$ cells. Mature white adipocytes are round shaped cells containing a single giant lipid droplet in the cytoplasm, few mitochondria, and a flattened, peripherally located nucleus. Mature brown adipocytes are polygonal cells with multiple scattered lipid droplets in the cytoplasm, and a round, eccentrically, yet not peripherally, located nucleus. Brown adipocytes contain more mitochondria, which are bigger in size and have more cristae than the ones in white adipocytes.

Table 1. Similarities and diversities of white, brown, and beige adipose tissue.

(Barneda *et al.*, 2013; Cedikova *et al.*, 2016; Kajimura *et al.*, 2015; Saely *et al.*, 2012; Sanchez-Gurmaches *et al.*, 2012; Seale *et al.*, 2008; Szentirmai & Kapas, 2017; Vargas *et al.*, 2015).

Features	White	Brown	Beige
Development	Mesenchymal stem cell; Predominantly Myf-negative precursor cells	Mesenchymal stem cell; Myf-positive precursor cells	Mesenchymal stem cell; Predominantly Myf-negative precursor cells
Develop time	Primarily after birth	At neonatal stage	Upon WAT development
Vasculature	Poorly developed Few capillaries	Highly developed	N/A
Nervous structure	Poor innervation	High innervation	N/A
Cell shape	Round	Polygonal	Round
Cell size	25µm ~ 200µm	15µm ~ 60µm	Variable depending on stimulation
Nucleus	Flattened, peripherally located	Round, eccentrically located	Depend on stimulation
Lipid droplets (LDs)	One giant LD	Multiple small LDs	Multiple variable sized LDs
Mitochondria	Few Thin and spindle	High amount Large and elliptical or round	Variable depending on stimulation
UCP1 expression	Undetectable	High	Variable depending on stimulation
Endoplasmic reticulum	Normal amount	Poor	Depend on stimulation
Main function	Fat storage	Thermogenesis	Adjust to cold exposure; thermogenesis
Location	Subcutaneous and visceral	Cervical, supraclavicular, paravertebral	Within WAT
Role in obesity	Positive	Negative	Negative

The discovery and study of beige (or brite) adipocytes came up quite recently when researchers observed the trans-differentiation of brown-like adipocytes from white adipocytes in WAT depots after chronic cold exposure (Barbatelli *et al.*, 2010). The first usage of the term “beige” to define the brown-like white adipocytes, to my knowledge, was in the comments to a published paper discussing *de novo* recruitment of brown adipocytes in 2010 (Vegiopoulos *et al.*, 2010; Ishibashi & Seale, 2010). The trigger of the browning process has not been fully elucidated but appears to be affected by the excitement of the SNS, levels of hormones, the tension of physical exercises, and time and extent of cold exposure (Poekes *et al.*, 2015). Many studies support the decisive role of β 3-AR in white adipocyte browning (Bartness & Ryu, 2015). But controversial views came up towards the necessity of the activation of β 3-AR, either by the norepinephrine or the β 3-adrenergic agonist, in the browning process. The passive role of β 3-AR is supported by evidence that the β 3-AR is dispensable for the browning process (de Jong *et al.*, 2017). Since the beige cells are transdifferentiated from the white adipocytes, it is predictable that they share some features in morphology and function with the white adipocytes, such as sharing the same precursors as well as the same locations (Table 1). Upon the browning process, the beige cells will show intermediate features between the two types of adipocytes and generally transform towards the brown ones. The changes include increased expression of UCP1 in mitochondria, increased number but decreased size of lipid droplets and mitochondria, increased sensitivity of β 3-AR, increased innervation and vascularization (Table 1). Recently, the beige cells attract a broad interest from

scientists as a result of its exercise- and temperature-inducible characteristics. More will be discussed in the next unit.

I.1.1.4 Current therapies of obesity: a long way to go

As one of the most severe and prevalent diseases raised recently, variable treatments towards obesity are offered for obese patients to choose from. Basic strategies of weight control include diet to control the total intake of calories, physical activities/exercises to consume energy, and behavior change/lifestyle therapies (Kushner, 2014). Multiple drugs, such as phentermine, orlistat, lorcaserin, and certain combinations such as phentermine/topiramate, functioning as either appetite suppressors or lipogenesis inhibitors, have been approved by the Food and Drug Administration (FDA) for the treatment of obesity. The most effective treatment for obesity may be bariatric surgery. However, only severe patients or patients with severe complications should be considered as surgery candidates. The decision to undergo surgery must be made on the basis of a comprehensive assessment evaluating both risks and benefits (Mechanick *et al.*, 2013). As a consequence of the low patient compliance with physical therapies, unavoidable side effects of current medicines, restrict applicability of surgery, researchers and doctors are still looking for effective and safe ways to treat obesity.

Several studies have been done to detect the potential possibility of recruiting and activating the BAT and beige cells in obese patients for weight control because of the negative role of BAT and beige in obesity. Gene transgenic mice with ablated BAT were associated with insulin resistance and non-insulin-

dependent diabetes mellitus, reduced rate of energy consumption, and considerable obesity even with a regular diet (Hamann *et al.*, 1988). Besides, human studies showed that the BAT activity is negatively related with BMI and body fat percentage, but was positively associated with cold-induced thermogenesis (Vijgen *et al.*, 2011; van Marken Lichtenbelt & Daanen, 2003). Though some *in vitro* studies showed that activating β 3-AR promotes lipolysis and thermogenesis of both brown and beige adipocytes as described above, the usage of β 3-AR agonists for obesity treatment received disappointing results in humans so far (S Qian *et al.*, 2015).

Bone morphogenetic protein 4 (BMP-4) agonist and BMP-4 inhibitors have recently been recommended as a potential medicine or drug target, respectively, for obesity treatment. An animal study showed that BMP-4 transgene mice exhibited a reduced WAT mass, decreased number of white adipocytes, and increased beige cells and SVF (SW Qian *et al.*, 2013). Conversely, the BMP-4-knockout mice had depressed UCP-1 expression, decreased mitochondria size, and increased lipogenesis along with diminished insulin sensitivity (SW Qian *et al.*, 2013). Human studies showed that silencing of Gremlin-1, a potent BMP-4 inhibitor, dramatically increased both beige and BAT markers, while the silencing of chordin and chordin-like-1, a BMP-4 binding protein, markedly reduced adipogenesis markers, such as peroxisome proliferator-activated receptor-gamma (PPAR γ) and fatty acid binding protein 4 (FABP4) (Gustafson *et al.*, 2015). However, since BMPs have a broad range of functions not only on adipocytes but also on

angiogenesis and bone formation, the usage of BMP agonists on obese treatment needs to be intensely studied before being used on humans.

Other factors like IGF-1, fatty acids, glucocorticoids, macrophage colony stimulating factor, and prostaglandins, have been identified as stimulators of the adipogenesis process (MacDougald & Mandrup, 2002). While some other factors, such as proteins from the Wnt family, transforming growth factor beta (TGF- β), interleukin 1 (IL-1), TNF- α , and epidermal growth factor (EGF), all showed inhibitory role in adipogenesis (MacDougald & Mandrup, 2002). However, it is difficult to use these intracellular or extracellular factors as targets since the stimulation or inhibition of them could result in complex cellular and systematic responses.

Quite recently, Gomisins N, a physiological lignan extracted from *Schisandra chinensis*, showed inhibitory effects on pre-adipocyte differentiation (Jang *et al.*, 2017). But whether this lignan functions appropriately *in vivo* and whether it will induce severe side effect in the human body remain unknown.

In summary, to find a thorough and secure treatment for obesity still requires more lab work and clinical tests to be done.

I.1.2 Process of adipogenesis *in vitro*: the lipids factory under precise regulation

Adipogenesis is the formation of mature adipocytes differentiated from fibroblast-like pre-adipocytes. Thus, when considering obesity from a molecular level, the essential cause could be viewed as a result of the formation and

accumulation of adipocytes both subcutaneously or/and viscerally. Besides, adipogenesis is a continuously happened normal physiological process that not only store lipids for energy expenses, but also regulate body metabolism and homeostasis.

The highly-controlled process of adipocyte differentiation, especially the white adipocyte differentiation, has been extensively studied in the past 30 years. Two types of *in vitro* experimental models have been used. One is the pre-adipocyte cell lines, such as the 3T3-L1 and the 3T3-F442A cell line, which is isolated from the Swiss 3T3 cell line. These pre-adipocyte cell lines only have the ability to differentiate into adipocytes. The other is the multipotent stem cell lines, such as the C3H10T1/2 cell line from C3H mouse embryonic stem cell precursors and the adipose-derived stem cells from human adipose tissue. These multipotent cell lines have the potential to commit to different lineages, including adipose, bone, and muscle lineage (Moreno-Navarrete & Fernández-Real, 2012).

To achieve a successful differentiation into mature adipocytes, morphology, transduction of signals, and protein expression of the pre-adipocytes change dramatically through a regulated process. All mature adipocytes are characterized by the existence and accumulation of big lipid droplets (comparing to other type of cells) in the cytosol, high expression of adipogenic transcription factors (such as PPAR γ and CCAAT/enhancer-binding proteins (C/EBPs)) and adipogenic markers (such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and fatty acid binding protein 4 (FABP4)). However, the stimulation of differentiation and the

adipogenesis process for the three types of adipocytes – the white, brown and beige cells – are not the same.

I.1.2.1 Lipids and lipid droplets in adipocytes

Lipid droplets (LDs) are lipid-rich cellular organelles that can be found in all eukaryotic organisms and even some prokaryotic cells (Grant & Donaldson). The formation of lipid droplets in the cytosol, with a variable range from less than 1µm to more than 100µm, is the main characteristic of mature adipocytes (Suzuki *et al.*, 2011). Thus, it is of great importance to understand the structure and metabolism of the LDs (Figure 2).

In adipocytes, LDs are observed as round structures under the microscope. The lipid droplets are isolated from other organelles and the aqueous intracellular environment by a monolayer of phospholipid embedded or adhered by several types of proteins, including the perilipin (Plin) family proteins (Plin1~5), the cell death-inducing DFF45-like effector (Cide) family proteins (Cidea, Cideb, Cidec), adipose triglyceride lipase (ATGL), and some membrane-trafficking proteins (Barneda *et al.*, 2013; Guo *et al.*, 2009). The Plins stabilize the lipid droplets and govern lipolysis. The Cides regulate the size of LDs. The adipose triglyceride lipase hydrolyzes the first fatty acid of triacylglycerol (TAG) to start lipolysis. Membrane-trafficking proteins, such as the Rab proteins, govern the fission and fusion of vesicles, deliver proteins to different cellular compartments, and regulate the signal transduction. With the phospholipid layer surrounded, the hydrophobic core of LDs in white adipocytes are predominately made up of triacylglycerols (TAGs) (Figure

2.A). Thus, the formation and growth of LDs require the production and transportation of the core lipids (mainly TAGs with a small amount of sterol esters), the surface phospholipid monolayer and the lipid-related proteins. Several models have been proposed to describe the life cycle of LDs, but none of the models was successfully and sufficiently supported by current data. The following introduction will build on studies conducted on 3T3-L1 pre-adipocytes cell line *in vitro* or/and mice/human adipose tissue *in vivo*.

Generally, the life cycle of the LDs starts with fatty acids. The fatty acids can diffuse into the cell through fatty acid transporter (FAT, CD36) or fatty acid transport proteins (FATPs, especially the FATP1) that are located on the cell membrane. The fatty acids can also be synthesized through *de novo* lipogenesis that takes place in the cytoplasm (Figure 2.B). *De novo* lipogenesis normally contributes about 20% of the total adipose TAG formation. But this *de novo* lipogenesis process is able to provide all necessary fatty acids for adipogenesis process (Strawford *et al.*, 2004; JM Collins *et al.*, 2011). The fatty acid transport protein type 1 (FATP1) is highly expressed on the membrane of both WAT and BAT initially (Schaffer & Lodish, 1994). Though insulin stimulation does not change the expression of FATP1, it facilitates the translocation of FATP1 to the plasma membrane from an intracellular compartment in the 3T3-L1 pre-adipocytes (Stahl *et al.*, 2002). During *de novo* lipogenesis, palmitate (C16:0) is constructed by continuously adding malonyl-CoA to acetyl-CoA (Figure 2). Two key enzymes are involved in the process: ACC and FAS (JM Collins *et al.*, 2011). First, acetyl-CoA, an intermediate product of glycolysis, is carboxylated into malonyl-CoA by ACC

(Wakil & Abu-Elheiga, 2009). Acetyl-CoA carboxylase (ACC) is a complex multifunctional biotin-containing enzyme system that performs the carboxylation reaction through two steps: 1) ACC acts as a biotin carboxylase in an ATP-dependent manner; 2) ACC acts as a carboxyl transferase to transfer the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA (Gurr *et al.*, 2016). Next, the condensation reaction is conducted by FAS to form 16-carbon palmitate using acetyl-CoA and malonyl-CoA as substrates (Currie *et al.*, 2013). The FAS is a giant multifunctional enzyme system formed by homodimer of two identical monomers (Berg *et al.*, 2015). Each monomer has seven covalently linked catalytic sites, which are divided into three domains, to produce palmitate (Chirala & Wakil, 2004). The chemically inert FAs, either diffuse into the cell or be generated endogenously, need to bind to cytosolic FABP4 and be esterified with CoA. The esterification is an ATP-dependent two-step reaction conducted by acyl-CoA synthetase (ACS) — to enter the bioactive pool in the form of fatty acyl-CoA (FA-CoA) that binds to the acyl-CoA-binding protein (ACBP) (Figure 2.B). Fatty acid binding protein 4 (FABP4) belongs to the FABP family and is highly expressed in adipocytes. It is a ~15 kDa small protein, which acts as intracellular lipid carrier and transporter, to regulate lipid trafficking and targeting (Furuhashi *et al.*, 2014).

The majority of TAGs are generated through the glycerol 3-phosphate (G3P) pathway at the endoplasmic reticulum (ER) membrane where the synthesis process is conducted by enzymes either permanently or transiently located (Pol *et al.*, 2014). More specifically, the TAGs are formed and deposited between the leaflets of the ER membrane (Kohlwein *et al.*, 2013). As the triose sugar backbone

of TAG, G3P is mainly generated through 3 metabolic processes: 1) gluconeogenesis, in which dihydroxyacetone phosphate is reduced to G3P by G3P dehydrogenase during glycolysis, 2) phosphorylation of glycerol via glycerol kinase, and 3) glyceroneogenesis, in which G3P is formed from non-glycerol/glucose precursors, such as pyruvate, amino acids, and lactate (Reshef *et al.*, 2003). Gluconeogenesis can be upregulated in obese individuals since the activity of G3P dehydrogenase from lean individuals is only half of that from the morbidly adipose tissue of obese individuals (Swierczynski *et al.*, 2003). Under insulin stimulation, the PPAR γ -mediated increased expression and activity of glycerol kinase allow glycerol to be a direct source of G3P (Guan *et al.*, 2002). However, by using the tritium ($[^3\text{H}_2]\text{O}$) to track body water, glyceroneogenesis was found to be the quantitatively dominant source of G3P in adipose tissue of rats (Nye *et al.*, 2008).

Triacylglycerol synthesis is the esterification of the alcoholic groups of G3P in a row. Several reactions happen successively and are conducted by specific enzymes, including G3P acyltransferase (GPAT), 1-acylglycerol-3-phosphate acyltransferase (AGPAT), phosphatidate phosphohydrolase (PAP), and diacylglycerol acyltransferase (DGAT) (Figure 2.B). First, GPAT and AGPAT transfer acyl groups to G3P stepwise. Then PAP catalyzed the dephosphorylation reaction, and DGAT converts the generated diacylglycerol (DAG) to TAG (Saponaro *et al.*, 2015). DGAT is one of the most important enzymes in the process. The expression and activity of DGAT can be upregulated by both insulin and

glucose. However, the deletion of DGAT in adipocytes leads to depleted DGAT activity, TG synthesis, and LD formation (Harris *et al.*, 2011; Meegalla *et al.*, 2002).

Figure 2. Structure and formation of lipid droplets (LD) in adipocytes.

A. LD structure. LD is observed as round structure with a hydrophobic core mainly made up of TAGs. LD is surrounded by a monolayer of phospholipid embedded or adhered by several types of proteins, including the Plins, the Cides, ATGL, Rab5, and Rab18. **B. Formation of LDs.** Fatty acids from the diet were either transported into adipocytes through FAT and FATP located on the plasma membrane or synthesized *de novo* in the cytosol from acetyl-CoA. The inert fatty acids will be activated by ACS to form FA-CoA. Then on the membrane of ER, TAG will be synthesized using G3P and FA-CoA as substrates via a chain of reactions mediated by GPAT, AGPAT, PAP, and DGAT.

(ACBP: acyl-CoA-binding protein; ACS: acyl-CoA synthetase; AGPAT: 1-acylglycerol-3-phosphate acyltransferase; ATGL: adipose triglyceride lipase; Cide: cell death-inducing DFF45-like effector; DAG: diacylglycerol; DGAT: diacylglycerol acyltransferase; ER: endoplasmic reticulum; FA-CoA: fatty acyl-CoA; FAT: fatty acid transporter; FATP: fatty acid transport protein; G3P: glycerol 3-phosphate; GPAT: glycerol 3-phosphate acyltransferase; PAP: phosphatidate phosphohydrolase; Plin: perilipin; TAG: triacylglycerol.) (JM Collins *et al.*, 2011; Eehalt *et al.*, 2006; Garrett & Grisham, 2017; Pol *et al.*, 2014; Gurr *et al.*, 2016; Fujimoto & Parton, 2011)

Though it is widely believed that the TAGs are synthesized between the leaflets of the ER membrane, how the neutral lipids and LD-associated proteins are assembled, how and whether the mature LDs separate from the ER membrane remain incompletely understood (Hashemi & Goodman, 2015). The exact pathways of LDs expansion and how much each possible pathway contribute to LDs expansion also remain unclear. Nevertheless, it is widely accepted that LDs can grow in size. One possibility is that the neutral lipids and phospholipids were transported to the LDs through LD-ER contact sites (Yang *et al.*, 2012). The assumption was supported by electron and immunoelectron microscopy data showing that the small LDs (around 1 μ m in diameter) were surrounded by several layers of ER (Heid *et al.*, 2014). Another possibility is that the neutral lipids and phospholipids were synthesized directly on the LDs membrane. This scenario is supported by localization of neutral lipids synthesis related proteins – such as GPATs, AGPATs, and DGATs – and phospholipid related proteins – such as CTP:phosphocholine cytidyltransferase (CCT) – on the LDs membrane (Guo *et al.*, 2008; Wilfling *et al.*, 2013). Fusion of smaller LDs into bigger LDs also seems to contribute to the growth of LDs. The fusion event is rare in normal conditions but happens quite often in the 3T3-L1 pre-adipocytes upon insulin treatment (Guo *et al.*, 2008; Ariotti *et al.*, 2012).

In summary, the formation of LDs is a multistep process that is conducted by a series of enzymes whose expression and activity can be regulated by extracellular stimuli – such as insulin and glucose.

I.1.2.2 Insulin, dexamethasone, 3-isobutyl-1-methylxanthine: the three musketeers

As is mentioned above, adipocyte differentiation is a highly regulated and complicated process in which numerous hormones, proteins, and signaling pathways function together and successively. Commonly, scientists mimic the trigger of pre-adipocyte differentiation by adding a hormone combination containing insulin, dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX) *in vitro*. These three “musketeers” work together to help d'Artagnan – the pre-adipocytes – undergoing a regulated differentiation process. Though how exactly these hormones regulate adipogenesis is not fully explored, some basic functions will be discussed below.

Insulin is a major regulator of cell metabolism and also the most potent hormone among the three “musketeers” to initiate adipogenesis (Klemm *et al.*, 2001). It is a hormone secreted by B cells of the pancreas and performs its function of regulating carbohydrate metabolism by activating the insulin receptors (IRs) and the IGF receptors (IGFRs) located on the plasma membrane (details of the IR, the IGFR, the insulin signaling pathways, and how the pathways affect adipogenesis will be discussed later in another section). The human circulating (biological active) insulin is a monomer made up of two chains: an A chain with 21 amino acids and a B chain with 30 amino acids (De Meyts, 2004). In general, insulin alters body metabolism by affecting glucose transport and glycolysis, fatty acids and TAG synthesis, the rate of protein synthesis, and the expression of adipocyte differentiation-associated proteins (Dimitriadis *et al.*, 2011).

The capability of insulin to govern the transportation of glucose, which is essential for adipocyte differentiation as the source of energy and TAGs, mostly relies on its regulatory role towards the glucose transporter type 4 (GLUT4). The GLUT4, an insulin-sensitive glucose transport protein, is uniquely expressed on the plasma membrane of adipocytes (James *et al.*, 1988). Under the normal state (without insulin stimulation), only a small portion of GLUT4s are found on the cell surface, while the majority of GLUT4s stay on specific intracellular compartments—the specialized GLUT4 storage vesicles (GSVs)—which locate peripherally close to the plasma membrane (Leto & Saltiel, 2012). Insulin augments the cell surface GLUT4 of adipocytes by increasing exocytosis of the GSVs and decreasing endocytosis of GLUT4 on the plasma membrane (Czech & Buxton, 1993; Jhun *et al.*, 1992). These GLUT4 transporters were endocytosed into the cell through both clathrin-mediated endocytosis and the cholesterol-dependent endocytosis (Lajoie & Nabi, 2010). During internalization, small GTPases (e.g., Rab5), guide the GLUT4-containing vesicles towards the cell interior (Huang *et al.*, 2001). Upon the stimulation of insulin, several intracellular signaling cascades are initiated, including the extracellular signal regulated kinase (Erk)1/2 signaling cascade, and the phosphatidylinositide 3-kinase (PI3K)/Akt signaling cascade (Zarich *et al.*, 2006). However, the former cascade has been found to be unimportant for glucose transportation (Lazar *et al.*, 1995). Instead, the insulin signaling pathway significantly increases the exocytosis of GSVs by controlling the activation states of small GTPases via activating the PI3K/Akt signaling cascade (Leto & Saltiel, 2012). Once is recruited and activated, Akt phosphorylates and inactivates two

GAPs, the AS160 – for vesicle translocation, and the RAL–GAP complex (RGC) – for vesicle targeting (Leto & Saltiel, 2012). Thus, insulin is able to regulate glucose uptake by increasing the GLUT4 level and the duration of GLUT4 remains on the plasma membrane. Besides, studies also show that the 3T3-L1 pre-adipocytes, once they reach confluence, become more sensitive to insulin stimulation. Under the pre-confluent state, the cell surface GLUT4 level of 3T3-L1 cells was increased by 70% upon insulin stimulation; while under confluent state, the surface transporter level of 3T3-L1 cells was increased by 270% upon insulin stimulation (Govers *et al.*, 2004).

Dexamethasone is a synthetic glucocorticoid that performs an anti-inflammatory role in the body. It triggers the differentiation of primary MSCs into osteocytes and adipocytes *in vitro*. With either an increased concentration of DEX in media or an extended incubation time with DEX, the MSCs prefer adipogenic differentiation than osteogenic differentiation (Yin *et al.*, 2006). 3-isobutyl-1-methylxanthine (IBMX) is one of the most potent and competitive, non-specific inhibitor of phosphodiesterases that enhance the intracellular cyclic adenosine monophosphate (cAMP) and activate protein kinase A (PKA) (Schroeder *et al.*, 2012). Since the PKA signaling pathway is required for the transcriptional activation of PPAR γ , IBMX performs a regulatory role in the gene expression during differentiation. A study has shown that treatment of IBMX on the 3T3-L1 cells does not drive the pre-adipocytes towards adipogenic differentiation. But that did not represent a final fate of the pre-adipocytes. Indeed, treatment of DEX on

IBMX pre-treated cells followed by an additional 48 hours of IBMX treatment triggered the differentiation process (Pantoja *et al.*, 2008).

In conclusion, the three hormones, insulin, DEX, and IBMX, ensure the 3T3-L1 pre-adipocytes to enter and complete the differentiation process properly. Insulin promotes the adipogenesis process by regulating the GLUT4 levels on the plasma membrane and the sensitivity of the cells to respond to insulin stimulation. Dexamethasone, in combination with IBMX, increase the expression of PPAR γ , C/EBP δ , and C/EBP β , which are important adipogenic transcription factors for adipocyte differentiation (Cao *et al.*, 1991).

I.1.2.3 Adipogenesis *in vitro*: the mitotic clonal expansion phase and the morphological change/terminal differentiation phase

As is mentioned above, two different kinds of *in vitro* models are used in the study of adipocyte differentiation: the pre-adipocyte cell lines and the multipotent stem cell lines. Some cell types undergo a proliferative phase called the mitotic clonal expansion (MCE) phase before terminal differentiation, while some other cell types directly enter the differentiation phase. Here, I will discuss the 3T3-L1 (pre-adipocyte cell line) adipogenesis process.

In the process of 3T3-L1 pre-adipocytes differentiation, insulin action, glucose uptake, lipid transportation and synthesis, transcription factors (e.g., PPAR γ and C/EBP α), cell cycle proteins (e.g., CDK2 (Cyclin-dependent kinase 2), cyclin D1 and retinoblastoma (Rb)), adipocyte-associated proteins (e.g., ACC and FAS), and activation of signaling cascades (e.g., the insulin signaling pathway) are

required (Koppen & Kalkhoven, 2010). Before hormone activation, the pre-adipocytes stop proliferation once they reach cell confluence. The treatment of hormone cocktail (combination of insulin, DEX, and IBMX) will trigger the cells to enter the fate of differentiation, which includes two phases: the MCE phase and the morphological change/terminal differentiation phase (Figure 3). The former phase consists of two rounds of post-confluence mitosis, and the latter phase, including the transition to a round cell shape from an elongated cell shape and to contain large globules of fat in the cytoplasm, is the morphological change of pre-adipocytes into adipocytes, (Figure 3).

In the MCE phase (usually occupies the first 48 hours after hormone treatment), a group of cell cycle proteins are periodically expressed and degraded to ensure proliferation through mitosis. Cyclin D1 is a protein expressed periodically in the G1 phase of the cell cycle, and it is required for the transition from G1 phase to S phase. cAMP elevates the expression of cyclin D1 during the first few hours of adipogenesis, which is induced by IBMX and insulin (Fox *et al.*, 2008). CDK2 belongs to a family of protein kinases that regulated the cell cycle. The expression of CDK2 is required during the G1/S transition because of its catalytic role in the cyclin-dependent protein kinase complex (Peng *et al.*, 2016). Retinoblastoma is phosphorylated by the cyclin-dependent protein kinase complex, which promotes transcriptional activation of the cell cycle required genes to drive the cells enter S phase (Berthet *et al.*, 2006).

Meanwhile, adipogenic specific proteins (e.g., C/EBPs and PPAR γ) are regulated transcriptionally, which will be discussed in the next section.

Figure 3. The process of adipogenesis.

Pre-adipocytes are fibroblast-like elongate-shaped cells with a round, centrally located nucleus. Upon the stimulation of hormone cocktail (combination of insulin, IBMX and DEX), pre-adipocytes undergo a ten-day process of differentiation named adipogenesis. Two phases are involved in this process. The clonal expansion phase, which happens in the first 48hs of adipogenesis, allows pre-adipocytes to go through two cycles of mitosis to quadruple the cell. In the latter terminal differentiation phase, lipid droplets will be generated, accumulated, and fused in the cytoplasm and eventually form a large lipid vacuole. (Rosen & Spiegelman, 2000)

I.1.2.4 The adipogenic markers: C/EBPs (C/EBP α , C/EBP β , and C/EBP δ) and PPAR γ

A series of changes happen simultaneously and successively in transcriptional level during adipogenesis (Figure 4). The C/EBP family members share the characteristics of C-terminal leucine zipper domain for dimerization and a DNA-binding domain for transactivation activity. Three of the family members, C/EBP $\alpha/\beta/\delta$, have established roles in adipogenesis. During the first 24 hours after

hormone treatment, a transient, fast, and dramatic accumulation of C/EBP β and C/EBP δ will appear within the MCE phase (Figure 4). The expression of C/EBP β is critical for adipogenesis, which is supported by a study showing that mouse embryo fibroblasts from C/EBP β ($-/-$) mice never enter the MCE phase and cannot differentiate into adipocytes (Tang *et al.*, 2003). Once is hyperphosphorylated by the mitogen-activating protein kinase (MAPK) and glycogen synthase kinase 3 β (GSK-3 β), C/EBP β acquires its DNA-binding activity and transactivate both C/EBP α and PPAR γ (Tang *et al.*, 2005). Though C/EBP β appears within 2 hours after hormone induction, it only gains DNA-binding activity after a long lag when the cells enter S phase during the mitotic clonal expansion phase (MCE) (Figure 4). An *in vivo* study shows that newborn C/EBP β ($-/-$) $\cdot$ δ ($-/-$) mice does not accumulate droplets, and ependymal fat pad weight of adult C/EBP β ($-/-$) $\cdot$ δ ($-/-$) mice is significantly reduced (Tanaka *et al.*, 1997). The growth of C/EBP δ deficient pre-adipocytes are dramatically depressed under hormone induction (Hishida *et al.*, 2009). However, the overexpression of C/EBP β , not C/EBP δ , is sufficient to induce adipogenesis, which indicates a regulatory, but less important role of C/EBP δ in adipogenesis (Yeh *et al.*, 1995). The expression of C/EBP β and C/EBP δ both peak shortly after hormone induction and experience a sudden decrease together with the expression of low levels of C/EBP α and PPAR γ . C/EBP α and PPAR γ can then initiate the expression of each other in a positive feedback loop, perform proliferation inhibition activity, regulate adipogenic genes and proteins, and remain continuously elevated during adipogenesis (Figure 4).

Figure 4. The regulation of transcription factors during adipogenesis.

A transient and dramatic increase of C/EBP β and C/EBP δ takes place within the first 2 hours after hormone treatment and experience a sudden decrease at the end of the mitotic clonal expansion (MCE) phase. The expression of these two transcription factors transactivates both C/EBP α and PPAR γ , which initiate the expression of each other in a positive feedback loop, when the cells cease the MCE phase. (Ramji & Foka, 2002; Rosen *et al.*, 2002).

The two key transcription factors, C/EBP α and PPAR γ , emerge and dramatically increase in the second wave (the C/EBP β and C/EBP δ are the first wave) of adipogenic transcription factors during the development of all types of adipose cells. A genomic study shows that PPAR γ binds to 5299 genomic regions, where over 60% are also found to be bound by C/EBP α in the 3T3-L1 pre-adipocytes (Lefterova *et al.*, 2008). Another study reports C/EBP α to be

responsible for insulin-sensitive glucose transport and the regulation of several adipogenic related proteins, such as diacylglycerol acyltransferase 1 and 2 (DGAT1/2) and ACC (Olofsson *et al.*, 2008; Z Wu *et al.*, 1999). Interestingly, PPAR γ expression is necessary and sufficient to induce adipogenesis even in the lack of C/EBP α in mouse embryonic fibroblasts. In concordance, C/EBP α itself is not able to induce adipogenesis in the absence of PPAR γ (Rosen & MacDougald, 2006). The expression of the dominant negative mutant of PPAR γ reduced the mRNAs of several adipogenic key enzymes – GLUT4, insulin receptor, and C/EBP α – in mature adipocytes (Tamori *et al.*, 2002). So far, no other transcription factors have been found to successively induce adipogenesis in the absence of PPAR γ , which indicates the importance and unique role of PPAR γ in this process. Peroxisome proliferator-activated receptor gamma (PPAR γ) exists in two isoforms, PPAR γ 1 and PPAR γ 2. The former one is more ubiquitously expressed while the latter one is exclusively expressed in adipocytes. Nevertheless, both isoforms are highly induced during adipogenesis (Siersbaek *et al.*, 2010).

In summary, the expression of PPAR γ and C/EBP α is induced by C/EBP β and C/EBP δ when the hormone-treated pre-adipocytes cease the MCE phase. Both PPAR γ and C/EBP α are essential for the differentiation of pre-adipocytes. However, only PPAR γ plays a unique and indispensable role in the differentiation process.

I.2 The receptor-mediated endocytosis

I.2.1 Endocytosis

All types of cells require fuel for metabolism. Eukaryotic cells, including the adipocytes, have the most complicated systems to communicate with the environment around them. Cells respond to changes and stimuli in the extracellular environment to mediate signal transduction, build interior trafficking structures, and secrete hormones to the extracellular environment (Alberts *et al.*, 2007). To complete these missions, eukaryotic cells developed a high-efficient internal membrane trafficking system to transfer proteins (such as cell-surface receptors) between different membrane structures and to take up solutes, particles, or macromolecules from the external environment. By the process of exocytosis, the cells deliver proteins, hormones, and lipids either to the plasma membrane or the extracellular environment. Through the contrary process named endocytosis, the cells internalize certain particles/macromolecules by surrounding them with an area of the plasma membrane, removing the plasma membrane from the cell surface to form a vesicle structure, and delivering the vesicle structure to different intracellular compartments in an energy-consuming manner (Cooper, 2000). Compartments, such as the ER and Golgi apparatus, in the exocytosis process have recognizable shapes and certain localizations. In contrast, compartments in endocytosis process exist as vesicles that are usually isolated from large cellular structures. As a result, two endocytic compartments, the early endosomes and the late endosomes, are distinguished by their localization and time of formation during the endocytic process (Kornilova, 2014). Endocytosis can generally be divided into

three stages. First, the cargos (solutes, particles, and macromolecules) are internalized into the cell with part of the plasma membrane as intracellular vesicles. Second, the internalized vesicles are sorted and delivered to the early endosomes and then to different intracellular membrane compartments. At last, the cargos will reach the final destination, either the lysosome to be degraded or the plasma membrane to be reused (Sahay *et al.*, 2010).

There are multiple pathways for the solutes, particles, and macromolecules to enter the cells. Endocytosis has been broadly classified as phagocytosis and pinocytosis (Cooper, 2000). These two categories can be distinguished by the size of material been internalized and also by the dependence of phagocytosis on actin polymerization as a key step in particle ingestion (J Liu & Shapiro, 2003). The process of taking large particles (generally larger than 250 nm in diameter), such as cell debris, bacteria or even another cell, is named phagocytosis (Doherty & McMahon, 2009). Specific cell types, such as macrophages, monocytes, and neutrophils are well-known “professional phagocytes” that wipe out foreign material (bacteria and pathogens) as part of the immune system. The uptake of fluids and solutes (such as vitamins, some ions, and cholesterol, which are generally less than 150 nm in diameter), together with the macromolecules (such as transmembrane receptors, ion-channels, and cell-surface transporters) that they bind to, is called pinocytosis (Cooper, 2000). Different from phagocytosis that usually occurs in phagocytotic cells, pinocytosis exists in all cell types. At least two classifications of pinocytosis have been proposed: one considers the material been engulfed into the cell; the other one emphasizes on the proteins that participate in

the process of endocytosis (Sahay *et al.*, 2010). The former one classified pinocytosis as receptor-mediated endocytosis, adsorptive endocytosis, and fluid-phase endocytosis (Khalil *et al.*, 2006). The fluid-phase endocytosis can occur by either micropinocytosis (vesicles less than 0.1 μm in diameter) or macropinocytosis (vesicles between 0.5 μm to 5 μm in diameter) (Kruth *et al.*, 2005). The latter one can further classify pinocytosis into two types: clathrin-dependent endocytosis, also known as clathrin-mediated endocytosis (CME), and clathrin-independent endocytosis (Sahay *et al.*, 2010).

I.2.2 Mechanism of receptor-mediated endocytosis: how the initial signal is implemented – magnification V.S. quenching.

I.2.2.1 The ligands and the receptor tyrosine kinases: the soulmates

Receptor-mediated endocytosis (RME), which is characterized by selective uptake of specific macromolecules followed by internalization of ligand-receptor complexes, generally occur through a clathrin-dependent process (Lodish *et al.*, 2016). The categories of macromolecules been internalized through RME are 1) peptide signaling growth factors, cytokines, and hormones, such as EGF, FGF, and insulin; 2) cell metabolism-related macromolecules, such as transferrin and low-density lipoprotein (LDL); 3) toxins, such as *Clostridium perfringens* Iota and *Clostridium botulinum* C2; 4) viruses, such as measles virus (through Nectin 4 receptor) and Rubella virus (through myelin oligodendrocyte glycoprotein, MOG); 5) bacteria, such as *Listeria monocytogenes* (through E-cadherin and Met) and *Yersinia pseudotuberculosis* (β 1-integrins) (Backovic & Rey, 2012; Cossart &

Helenius, 2014; Gibert *et al.*, 2011; Kornilova, 2014). I will only discuss the entry of macromolecules from the first category in this chapter.

Receptor-mediated endocytosis is controlled and regulated by a large number of specialized proteins to mediate fission and fusion of phospholipid membranes, engulfment of ligands and ligands binding proteins (the receptor tyrosine kinases, RTKs), movement and directing of ligand-receptor complexes containing vesicles towards different cellular structures (Doherty & McMahon, 2009). The process of RME starts with the binding of the macromolecules and specific RTKs, which are a group of cell-surface receptors that consist of 58 receptors divided into 20 subfamilies (Critchley *et al.*, 2018). Receptors and ligands come in matched pairs. Each RTK only recognizes one or a few specific ligand(s), for instance, the insulin receptor (IR) specifically identifies insulin and insulin-like growth factors (IGFs), the epidermal growth factor receptor (EGFR) binds to EGF and transforming growth factor α (TGF- α), and fibroblast growth factor receptor 4 (FGFR4, from the FGFR family) has high affinity to several ligands, including FGF-1, 2, 4, 6, 8, 9, 16, 17, 18, and 19 (Lodish *et al.*, 2016; Tiong *et al.*, 2013). Meanwhile, a ligand can be picked up by only one or a few receptor(s), e. g., insulin can be recognized by both the IRs and the IGF receptors (IGFRs), FGF-1 can binds to FGFR1/2/3/4 (Sorkin & Puthenveedu, 2014; Tiong *et al.*, 2013).

However, the binding affinities between different ligands towards the same receptor or different receptors with the same ligand are quite diverse. In summary, despite the fact that one RTK can recognize one or several ligand(s) and one

ligand can bind to different RTKs with vary affinities, the bindings between ligands and RTKs are of high specificity and precision.

I.2.2.2 The internalization of ligand-receptor complexes: engulfment does not mean suppression

The binding of RTKs with specific ligand(s) will form the ligand-receptor complexes, which will be internalized into the cell with part of the plasma membrane where the RTKs locate through RME (Figure 5.B). The responsiveness of cells to the extracellular signal molecules depends on the number of ligand-receptor complexes formed on the plasma membrane. Thus, the endocytosis of these complexes, which alters the distribution and concentration of RTKs on the plasma membrane, regulates the long-term homeostasis and sensitivity of the cell to specific ligands(s) (Lodish *et al.*, 2016).

Once the RTKs recognize the extracellular signal and form the ligand-receptor complexes, the area of the plasma membrane that contains these complexes will be pinched off as clathrin-coated pits (Figure 5). The internalization process requires the participation of two proteins, the adaptor protein 2 (AP2) and clathrin. The former one, AP2 complex, which is formed by 4 subunits (the large α and β 2 subunits, the medium-sized μ 2 subunit, and the small-sized σ 2 subunit) as a heterotetrameric structure, serves as bridges between the plasma membrane, RTK, and clathrin (Higgins & McMahon, 2002) Park, 2014 #1013). Knockout of AP2 in mice is embryonic lethal, indicating the crucial role of AP2 in embryonic development and cell homeostasis (Park & Guo, 2014). The latter one, clathrin,

has a triskelion structure with three clathrin heavy chains (CHC) stretched out from a central vertex that is made up by three tightly associated clathrin light chains (CLC) (Conner & Schmid, 2003; Royle, 2006). Knockout of the CHC in Hela cells results in diminished growth rate and impaired endocytosis (Hinrichsen *et al.*, 2003). To form the clathrin-coated pits, the first step is to “mark” the transmembrane receptors – the majority of which are RTKs – to be internalized. The marking is accomplished when cytoplasmic-located specific motifs of RTKs become exposed because of structural change under ligand binding (Pearse *et al.*, 2000). The well-characterized motifs include the tyrosine-based YXX Φ /NPXY motif and the dileucine-based [D/E]XXXL[L/I] motif (X is any amino acid, and Φ is a bulky hydrophobic amino acid, e.g., leucine, isoleucine, methionine, valine, and phenylalanine) (Bonifacino & Traub, 2003). The AP2 complex interacts with phosphatidylinositol-4,5-bisphosphate (PIP2) and phosphatidylinositol-3,4,5-triphosphate (PIP3) through its α subunit and μ 2 subunit. The interactions between the AP2 complex and PIP2/PIP3 are responsible for its localization on the plasma membrane (BM Collins *et al.*, 2002). The AP2 complex also recognizes the exposed YXX Φ /NPXY motif through the μ 2 subunit and the [D/E]XXXL[L/I] motif through μ 2 and/or β 2 subunit (Bonifacino & Traub, 2003). After binding to motifs located on the RTK, the AP2 complex undergoes a large conformational change from a locked state to an open state (Park & Guo, 2014). In the open state, AP2 complex recruits clathrin to the plasma membrane through the “hinge” domains of its large α and β 2 subunits and promotes the self-assembles of clathrin to build cage-like structures/lattice (Park & Guo, 2014). Hence, the curvature of the

membrane region that contains ligand-receptor complexes, the AP2 complexes, and the recruited clathrin is called the clathrin-coated pit. Through the action of other factors, such as endophilin and dynamin, the clathrin-coated pit further curves into the cell and finally will pinch off from the cell membrane (Figure 5.B).

The mature pits – the pits dissociate entirely from the cell membrane – will be uncoated and fuse to an early endosome where the ligand-receptor complexes are sorted to lysosomes to be degraded or recycled back to the plasma membrane to be reused (Figure 5.B). The ratio between the recycled receptors and degraded receptors varies. For instance, the insulin receptors in the 3T3-L1 pre-adipocytes are efficiently recycled upon insulin stimulation at a concentration of 100ng/ml (Marshall, 1985). High concentration of EGF (over 100ng/ml) promotes the degradation of EGFR while low concentration of EGF (lower than 50ng/ml) favors recycling of EGFR (Sigismund et al. 2008).

The role of RME involves both the rapid internalization of receptors following ligand binding and the subsequent sorting of the internalized ligand-receptor complexes (Goh & Sorkin, 2013). Thus, RME plays an essential role in controlling the functional activities of the RTKs.

Figure 5. The tyrosine kinase domain of RTKs and receptor-mediated endocytosis.

A. The tyrosine kinase domain of RTKs contains a smaller N-lobe and a bigger C-lobe. The cleft between the two lobes permits a specific site for ATP binding. B. Receptor tyrosine kinases are activated by binding with specific ligands followed by the formation of ligand-receptor complexes. The activated RTKs interact with AP2, which links to the plasma membrane and recruits clathrin. The ligand-receptor complexes then cluster on the plasma membrane to form a growing curved clathrin-coated pit. The mature pit then buds off from the plasma membrane and becomes the clathrin-coated pit (CCP). The CCP will be uncoated in the cytosol and the uncoated vesicle will be delivered to early endosomes (EEs) to fuse with them. Some ligand-receptor complexes remain intact while some ligands dissociate from the receptors when reaching the early endosomes. The ligands and some of the receptors will be sorted to late endosomes and eventually, lysosomes to be degraded. However, some of the receptors will be recycled to the plasma membrane. (Sorkin & Puthenveedu, 2014; Casem, 2016; Kirchhausen *et al.*, 2014; Cooper, 2000; Lemmon and Schlessinger, 2010).

As is mentioned above, the RME mediates the internalization and sorting of the RTKs. It is believed that the primary role of RME is to terminate the RTK signaling through ubiquitination and degradation upon vesicle sorting. However, several studies showed that the internalized ligand-receptor complexes continue functioning and mediate the full biological functions of the RTK signaling (von Zastrow & Sorkin, 2007). In other words, instead of just functioning as a delivery station, the endosomes that contain ligand-receptor complexes may also function as active signaling sites (Irannejad *et al.*, 2015). For instance, activated EGFR has been found to co-localize with endosomes and downstream signaling molecules, including the adaptor protein – Src homology and collagen protein (Shc, Src stands for *Rous sarcoma* oncogene cellular homolog), the Ras guanine nucleotide exchange factor (GEF) – son of sevenless (SOS), and the docking protein – growth factor receptor-bound protein 2 (Grb2) – *in vivo* (Di Guglielmo *et al.*, 1994). Besides, the internalized non-phosphorylated EGFR-ligand complex, which is induced by reversible inhibitors, could be activated in endosomes upon inhibitor wash-out and were able to perform its biological function by recruiting downstream signaling molecules (Pennock & Wang, 2003). Furthermore, the tropomyosin receptor kinase A (TrkA) receptors, once internalized upon nerve growth factor (NGF) binding, localize on the endocytic vesicles, remain phosphorylated, and are associated with phosphoinositide phospholipase C (PLC- γ 1), which acts as an essential intracellular signaling transduction enzyme that catalyzes the formation of inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (Grimes *et al.*, 1996). The TrkA-NGF complexes were also shown to move from the distal axon to the

neuronal cell body to carry the signal from the cell periphery to the nucleus (Cosker & Segal, 2014). Researchers also observed an accumulation of activated insulin receptors in the intracellular compartments in the presence of insulin (Kublaoui *et al.*, 1995).

In summary, the RTKs are internalized into the cell in the form of ligand-receptor complexes through clathrin-mediated endocytosis. The engulfed receptors, in some cases, are not degraded immediately. Instead, these receptors appear to remain activated and functional in endosomal compartments after internalization.

I.2.2.3 The small GTPase Rab proteins in the receptor-mediated endocytosis: the logistic company to control the delivery

As is mentioned above, common procedures of RME include the budding of clathrin-coated pits from the plasma membrane into the cell, movement of vesicles in the cytosol, targeting of the vesicles to specific destinations, and eventually the fusion of vesicles to subcellular structures (Gonzalez & Scheller, 1999). Numerous of proteins, including the Rab (Ras-related GTP-binding protein) family proteins, participate and act in the RME process to ensure the internalization, targeting, and fusion to take place precisely. The Rab proteins are a group of small GTPases (~20-30 kDa) belonging to the Ras superfamily and take part in the process of membrane trafficking (Grosshans *et al.*, 2006). Over 70 Rab proteins are found to function in intracellular transport and are classified into 6 groups based on their function in different components of intracellular membrane

trafficking, such as vesicle budding, delivery, fusion, and localization of certain proteins on different membrane domains (Stenmark, 2009). Mutations and abnormal expression of Rabs have been found to relate with many diseases. For instance, mutations on the Rab27a gene are responsible for the Griscelli syndrome (Menasche *et al.*, 2000). The G19V mutation of Rab38 leads to the Hermansky-Pudlak syndrome (Loftus *et al.*, 2002). Rab25, which belongs to the Rab11 subfamily, is elevated expressed and has an oncogenic function in ovarian, breast, prostate, bladder, and gastric cancer (S Wang *et al.*, 2017). Rab23, a negative regulator of the hedgehog signaling pathway that predominantly locates on the plasma membrane, has increased expression in hepatocellular carcinomas, diffuse-type gastric cancer, and follicular adenoma type of thyroid tumors (Evans *et al.*, 2003; Lim & Tang, 2015).

The Rab proteins function as molecular switches as they can be “turned off” to their GDP-bound form (inactive form) by GTPase activating proteins (GAPs) and “turned on” to their GTP-bound form (active form) by guanine-nucleotide exchange factors (GEFs) (G Li, 2015). By recycling between the two forms, the Rab proteins govern the receptor-mediated endocytosis by regulating membrane trafficking system, specifically, by recruiting Rab effector proteins to specific sites of the intracellular membrane structures. Every Rab protein has its unique localization on distinct membrane compartments to control the specificity of membrane structures and directionality of membrane trafficking (Zhen & Stenmark, 2015).

The Rab proteins can be found both in the cytosol and on membrane structures. The Rab GDP dissociation inhibitor (GDI) specifically recognizes the

GDP-bound Rabs. Thus, GDI solubilizes the Rabs and removes inactive Rabs from membrane structures (Figure 6). The GDI displacement factor (GDF), which locates on the donor membrane, initiates the recruitment of Rabs by dissociating the GDI from Rabs and inserting the Rabs into the membrane of intracellular compartments (Figure 6). To gain the membrane association ability, Rabs require post-translational modification of two cysteine residues located on or near the C-terminal (Leung *et al.*, 2006). The Rab proteins are then activated by a group of membrane-bound proteins named guanine nucleotide exchange factors (GEFs) (Figure 6). The activated GTP-bound Rabs then recruit several specific Rab effectors to facilitate vesicle budding, vesicle movement/delivery, and vesicle tethering (Muller & Goody, 2018). For instance, Rab1 and Rab2 are found on ER and *cis*-Golgi to regulate ER-Golgi and Golgi-ER trafficking, respectively; Rab5 is found to locate on early endosomes, clathrin-coated vesicles, and the plasma membrane to mediate vesicle budding and early endosome fusion; Rab7 is found on late endosomes to regulate late endosome trafficking (Stenmark & Olkkonen, 2001). Then the activated GTP-bound Rabs are converted to the inactive forms through hydrolysis of GTP, which is mediated by another group of proteins termed GTPase activating proteins (GAPs). As an end, the inactivated Rabs will be removed from the acceptor membrane by the GDIs to enter the cytosol.

Figure 6. Rab GTPases cycle.

Rab GTPases exist both in cytosol and on membrane. GDF, which locates on the donor membrane, displaces GDI and inserts Rabs onto the donor membrane. GEFs, which also locate on the donor membrane, replace the GDP to GTP to activate Rabs. Activated Rabs can recruit specific Rab effectors to mediate different membrane trafficking processes. The activated GTP-bound Rabs are converted to the inactive forms through hydrolysis of GTP, which is mediated by GAPs. Then GDIs specifically recognize the GDP-bound Rabs and thus solubilize the Rabs and remove inactive Rabs from the acceptor membrane. (GDI: Rab GDP dissociation inhibitor; GEF: guanine nucleotide exchange factor; GAP: GTPase-activating protein; GDF: GDI displacement factor). (G Li, 2015; Zhen & Stenmark, 2015)

In conclusion, the small GTPase Rab proteins perform their functions in the RME process by switching between their active and inactive forms and recruiting specific Rab effectors to certain membrane compartments.

I.2.3 Structure of the receptor tyrosine kinases

The human genome encodes 58 RTKs divided into 20 subfamilies, and the majority of RTKs share similar structures (Karpov *et al.*, 2015). Generally, an RTK is comprised of an extracellular domain for ligand binding, a single α -helix transmembrane domain, and an intracellular region that contains a juxtamembrane domain, a tyrosine kinase domain (TKD), and a C-terminal (Lemmon & Schlessinger, 2010). The extracellular domains of RTKs are quite diverse between different subfamilies, which ensures the high specificity of each RTK to recognize unique ligand(s) (Karpov *et al.*, 2015). On the contrary, the intracellular regions are relatively conserved between different subfamilies. The tyrosine kinase domain of RTKs has an N-lobe and a C-lobe (Figure 5.A). The smaller N-lobe consists of a five-stranded β -sheet and one α -helix, while the bigger C-lobe mainly consists of α -helices (Huse & Kuriyan, 2002). Besides the big cleft between the two lobes that forms a specific site for ATP binding, a phosphate-binding loop, which contains a highly conserved Gly-rich (GXGXXG) motif, poses the γ -phosphate of ATP and allows ATP to fit beneath the loop (Taylor & Kornev, 2011). Another highly conserved construction, the activation loop, which usually contains around 20 to 35 residues and is the primary site of regulatory phosphorylation, starts at the tripeptide Asp-Phe-Gly (DFG) motif and ends at another tripeptide Ala-Pro-Glu

motif (Svensson *et al.*, 2016; Bayliss *et al.*, 2015). The capacity of undergoing large conformational change allows the activation loop to switch between inactive and active states. In the inactive state, each TKD is stabilized in a unique auto-inhibition configuration by several particular intramolecular interactions between the juxtamembrane domain/C-terminal and the TKD (Lemmon & Schlessinger, 2010). Though the structures of inactive TKDs differ from receptor to receptor, which determines the diverse functions of RTKs, the active structures of all TKDs are very similar to each other (Nolen *et al.*, 2004). Upon activation of TKD by specific ligands, the phosphorylated tyrosine residues in the activation loop then serve as sites for assembly of signaling molecules that contain Src homology 2 (SH2) or phosphor-tyrosine-binding (PTB) domains (Axel Ullrich & Schlessinger, 1990). The phosphorylation of these signaling molecules can be triggered either directly by phosphorylated RTKs themselves, or indirectly by docking proteins, which are recruited and phosphorylated by activated RTKs (Schlessinger & Lemmon, 2003). Then the recruited signaling molecules pass down the initial signal and regulate cell survival, proliferation, and differentiation.

I.3 The insulin signaling pathways: the essential determinant of adipogenesis

The insulin receptor (IR) and insulin-like growth factor receptor 1 (IGF-1R) belong to the IR subfamily of RTKs. Both IR and IGF-1R have different affinities to insulin and IGFs (IGF-1/-2), which are from the same structural-related insulin-peptide superfamily (De Meyts *et al.*, 2000-2013). The insulin signaling pathways

that are triggered by the activation of the IR and IGF-1R integrate the storage and release of nutrients through human lives. The outlines of the insulins signaling pathways have been well described, but many details of signal transduction still need to be elucidated. The two well-established and most essential signaling pathways been initiated by insulin/IGFs are the Erk1/2 signaling pathway and the PI3K/Akt signaling pathway (Zarich *et al.*, 2006). The effects of insulin signaling pathways activation on adipocytes can be divided into three types: immediate effects, mid-term effects, and long-term effects. Immediate effects, such as the phosphorylation and dephosphorylation of specific enzymes and proteins, take place within several seconds after IR activation. Mid-term effects include the up- and down-regulated expression of specific proteins within 5-60 minutes after insulin stimulation. Long-term effects, e.g., cell proliferation and cell differentiation, will be detected from several hours to several days after insulin stimulation. Details of the effects of the insulin signaling pathways on adipocytes will be discussed later in this section.

I.3.1 Insulin receptor and its structure

The initiation of the insulin signaling pathways start with the binding of insulin or IGFs to the IR or the IGF-1R. Between the two receptors, IR is the primary site for insulin binding. The IR was first identified in 1971 by Cuatrecasas on adipocytes (Cuatrecasas, 1971). Through decades of research, the IR is proved to exist on the membrane of all types of mammalian cells with vary numbers from ~40 on erythrocytes to $\sim 200\sim 300 \times 10^3$ on adipocytes (Watanabe *et al.*, 1998).

Thus, dysregulation of cell metabolism happens when the IR is abnormally expressed on the cell surface. For instance, increased expression of the IR is often overserved in malignant cells. About 80% of the breast cancer cells have a significant higher IR content than the normal breast tissue cells. Besides, a higher level of IR expression predicts a poor survival in breast cancer patients (Vigneri *et al.*, 2016). The IR knockout mice only survived a few days after birth. The “fat-specific” IR knockout mice and adipocyte-specific IR knockout mice, though had a reduced fat mass and were protected from obesity-associated glucose intolerance, both suffered increased TAG accumulation in liver, hepatomegaly, and decreased lifespan (M. Bluher *et al.*, 2002; Eguchi *et al.*, 2011; Joshi *et al.*, 1996).

As a member of the RTKs, the IR contains an extracellular domain for ligand binding, a transmembrane domain, and an intracellular region which includes a juxtamembrane domain, a tyrosine kinase domain, and a C-terminal (Figure 7). However, unlike the prototypical RTKs that exist as single-chain polypeptides, IR exists as a covalently-linked tetramer consists of two α -subunits and two β -subunits (Weiss *et al.*, 2014). The four subunits are disulfide-linked to form a ~350 kDa structure. The α -subunit and 194 residues of the β -subunit together form the extracellular portion of the IR, while the rest of the β -subunit, which contains a single transmembrane part and a cytoplasmic portion (IR- β -tail) that permits the tyrosine kinase activity of the IR, is the intracellular portion (Belfiore *et al.*, 2009). Upon ligand binding, the α -subunits of the IR will inhibit the kinase activity of the β -subunits by inducing a structural change of the IR molecule at first. Then, this conformational change of the IR molecule will bring the two β -subunits close

enough to each other to permit the kinase activity of the β -subunits (Boucher *et al.*, 2014). The activation of tyrosine kinase activity of the IR includes the autophosphorylation of three tyrosine residues, which are Y1158, Y1162 and Y1163 in the activation loop of the kinase domain (contains ~300 residues) (Figure 7). Among the three tyrosine residues, the Y1162 is hydrogen-bonded to the catalytic loop that occludes the ATP-binding site in the absence of Mg^{2+} -ATP and will be the first to undergo autophosphorylation during IR activation, followed by autophosphorylation of the other two residues (Wei *et al.*, 1995). Mutations in these three residues not only affected the kinase activity of the IR but also impaired the biological function of the IR (Wilden *et al.*, 1992). Besides the three tyrosine residues that permit the catalytic kinase activity, some other tyrosine residues, such as Y965 and Y972 in the juxtamembrane region (~35 residues; between the transmembrane helix and the tyrosine kinase domain) and Y1328 and Y1334 in the C-terminal (~70 residues) of the β -subunit, are also autophosphorylated upon ligand binding (Figure 7). Mutations on the tyrosine sites leads to decreased signaling transduction and/or diminished insulin sensitivity, glycogen synthesis, and nutrients uptake (Table 2).

The IR has two isoforms: the IR isoform A (IR-A) without the exon 11, which codes a 12-amino acid segment at the C-terminal of α -subunit, and the IR isoform B (IR-B) with the exon 11 (Ebina *et al.*, 1985; A. Ullrich *et al.*, 1985). The two isoforms exhibit vary distribution in a tissue-specific manner and distinct cellular functional properties (Figure 7).

Figure 7 The insulin signaling pathway.

Insulin receptor (IR) is activated upon insulin binding. The activation of IR leads to the phosphorylation of several tyrosine kinases in the intracellular region of IR, including Y972 in the juxtamembrane region, Y1158, Y1162 and Y1163 in the tyrosine kinase domain and Y1328 and Y1334 in the C-terminal. The pY972 is responsible for the activation of IRS-1 and Shc through the phosphotyrosine-binding domain (PTB domain), while Y1158, Y1162 and Y1163 activate IRS-2 after phosphorylation. In the PI3K/Akt pathway showing on the right of the figure, the activated IRS-1/2 can directly recruit the PI3K and eventually lead to the activation of Akt. Glucose uptake, glycogen synthesis and Glut4 translocation are up-regulated upon Akt activation. In the Erk1/2 signaling pathway showing on the left, activated Shc recruits the Grb2-SOS complex, which mediates the GDP/GTP exchange of Ras. At the end, Erk1/2 will be activated and anticipates in the regulation of cell growth, gene expression and protein translation. (Cieniewicz *et al.*, 2016; Papich & Riviere, 2009; Youngren, 2007; Taguchi & White, 2008; Boucher *et al.*; Hubbard, 2013).

The IR-B has high affinity to insulin and is predominantly expressed in differentiated tissues that contain a higher number of IRs on the cell surface and have an inadequate response to IGF-2, such as liver, kidney, adipose tissue, and muscle. In contrast, though the IR-A has similar affinity to insulin as the IR-B, it exhibits high affinity to IGF-2. Besides, the IR-A is primarily expressed in fetal and tumor tissues with comparatively greater rates of internalization and recycling than the IR-B (Escribano *et al.*, 2017). Because of these differences, the IR-A activation is mainly associated with cell growth, cell proliferation, and cell survival, whereas the IR-B activation favors regulation of metabolism and differentiation. In the 32D cells, the IR-A preferentially sends mitogenic, antiapoptotic signals and translocate insulin receptor substrates (IRS-1) to the nucleus more effectively while the IR-B tends to send differentiation signals (Sciacca *et al.*, 2003). In vascular smooth muscle cells, the overexpression of IR-A confers a stronger migratory capability (Beneit *et al.*, 2016). The IR-A is also proved to be more efficient at increasing glycogen synthesis and storage and promoting glucose uptake than the IR-B (Diaz-Castroverde *et al.*, 2016).

The activation of the IR will induce the recruitment of downstream proteins that contain either a phosphotyrosine-binding domain (PTB domain) or an SH2 domain (Figure 7). E.g., IRS-1/-2 and Shc (Src homology and collagen protein) have a PTB domain, while Shc and APS (adaptor protein with pleckstrin homology and Src homology 2 domains) contain an SH2 domain (Boucher *et al.*, 2014; Taguchi & White, 2008). The PTB domain-containing proteins bind to pY972 (phosphorylated-Y972), which creates a lower affinity docking site for the PTB

domain of IRS-1, but a much higher affinity docking site for the same domain of Shc (Chaika *et al.*, 1999; SawkaVerhelle *et al.*, 1996). Interestingly, the Y972 belongs to the NPXY motif where the phosphorylation on the tyrosine site recruits the AP2 complex. In addition, pY972 is also the recruiting site for IRS-2. However, different from the IRS-1 that only interacts with the IR through one phosphorylated tyrosine (the pY972), the IRS-2 also interacts with the three tyrosines that located in the activation loop (Jensen & De Meyts, 2009; He *et al.*, 1996). As consequences of IR activation, two major signaling pathways are activated: the Erk1/2 signaling pathway and the PI3K/Akt signaling pathway (Figure 7). The specific function and regulation of the two pathways will be discussed later.

I.3.2 Insulin-like growth factor receptor 1 and its structure

The complementary DNA for human IGF-1R was cloned and sequenced in 1986 (A. Ullrich *et al.*, 1986). It binds to IGF-1 with higher affinity than IGF-2 but does not recognize insulin efficiently. The IGF-1R is widely expressed in many cell types in fetal and postnatal tissues and plays a role in essential cellular processes, including cell growth, cell differentiation, and apoptosis (Arcaro, 2013). Mice with null mutants for the IGF-1R exhibit a severe growth deficiency (only 45% of normal size) and die invariably at birth because of respiratory failure (JP Liu *et al.*, 1993). Non-malignant cells treated with IGF-1R nuclear translocation inhibitor or IGF-1R inhibitor display a decreased proliferation and migration rate than the untreated cells (Solomon-Zemler *et al.*, 2017). In addition to the role of the IGF-1R during development and cell proliferation, evidence also suggests a vital role of it in

malignant transformation (Sachdev & Yee, 2007). For instance, data show that the increased expression of the IGF-1R is associated with malignancy behavior in familial pheochromocytomas and paragangliomas regardless of the age at diagnosis and the genetic aetiology (Fernandez *et al.*, 2013). Evidence also shows that a disrupted activation of the IGF-1R results in inhibited growth and motility of a wide range of cancer cells *in vitro* and in mouse models (Sachdev & Yee, 2007).

The IGF-1R and the IR are from the same subfamily of RTKs. Thus, the IGF-1R is highly structural homologous to the IR, sharing 45% to 65% amino acid identity in the ligand-binding domain, 65% to 85% identity in the tyrosine kinase and substrate recruitment domains, and nearly absolute conservation in the ATP binding pocket (Escribano *et al.*, 2017). However, unlike the IR gene that is alternatively spliced to produce two isoforms, the IGF-1R gene is assembled without alternative splicing (Morris F. White, 2012). Similar to the IR, the IGF-1R is a tetramer consists of two α -subunits and two β -subunits. One α subunit and one β subunit form an $\alpha\beta$ half-receptor monomer. Two monomers are assembled into heterotetramer complex through disulfide bonds, which can be induced by treatment of IGF-1 or Mn/MgATP *in vitro* (Treadway *et al.*, 1989). Activation and signaling transduction of the IGF-1R is also quite similar to those of the IR. The three tyrosine residues locate on the activation loop of the tyrosine domain of IGF-1R are Y1131, Y1135, and Y1136 (these three tyrosine residues resemble the functions of Y1158, Y1162, and Y1163 on the IR). The Y950 locates on the NYXP motif of the juxtamembrane domain of the IGF-1R has similar function as the Y972 of the IR (Table 2).

Table 2. Locations and functions of essential amino acids on the β -subunit of the IR and the IGF-1R.

A. Locations of essential amino acid residues on the β -subunit.

Receptor	Full Length	Juxtamembrane Domain	Tyrosine Kinase Domain	C-Terminal
IR-A ¹	1343	Y953, Y960	K1018, Y1146, Y1150, Y1151	Y1316, Y1322
IR-B ²	1355	Y965, Y972	K1030, Y1158, Y1162, Y1163	Y1328, Y1334
IGF-1R ³	1337	Y943, Y950	K1003, Y1131, Y1135, Y1136	Y1316

¹ The numbering of amino acids of IR-A corresponds to the receptor of Ullrich *et al.* (A. Ullrich *et al.*, 1985).

² The numbering of amino acids of IR-B corresponds to the receptor of Ebina *et al.* (Ebina *et al.*, 1985).

³ The numbering of amino acids of IGF-1R corresponds to the receptor of Ullrich *et al.* (A. Ullrich *et al.*, 1986).

B. Functions of essential amino acid residues on the β -subunit of the IR-B.

Amino acid	Function
Y965	IR autophosphorylation site AP2 complex binding site ¹⁵
Y972	IR autophosphorylation site AP2 complex binding site ⁹ PTB domain-containing protein binding site ¹² IRS-1/2 binding site ⁵
K1030	ATP binding site ⁶
Y1158, Y1162, Y1163	IR autophosphorylation sites Essential tyrosine residues on the activation loop ¹⁴ IRS-2 binding site ⁹
Y1328, Y1334	IR autophosphorylation sites

⁵(He *et al.*, 1996); ⁶(M. F. White *et al.*, 1988); ⁹(Bonifacino & Traub, 2003); ¹²(Chaika *et al.*, 1999); ¹⁴(Cieniewicz *et al.*, 2016); ¹⁵(Y Xu *et al.*, 2011).

C. Effects of essential amino acid residue mutants on the β -subunit of the IR-B.

Mutant	Effects of Mutant
Y965F	No effect on IR internalization or IR auto-phosphorylation ¹¹ No interaction with IRS-1 ^{4,5} No effect on IRS-2 binding ⁵
Y972F	No effect on IR auto-phosphorylation ¹¹ Decreased insulin sensitivity ⁶ and rate of glucose transport ¹² Diminished glycogen synthesis and internalization ⁸ No effect on IRS-2 binding ⁵
Y965A	No effect on IR auto-phosphorylation ¹¹ Decreased IR internalization ¹¹
Y972A	No effect on IR auto-phosphorylation ¹¹ Decreased IR internalization ¹¹
Δ 966-977	No effect on auto-phosphorylation of β -subunit ⁸ Diminished downstream signaling, glycogen synthesis, and IR internalization ⁸
K1030A	No interaction with IRS-1/2 ^{4,5} Decreased IR internalization ¹³
K1030R	Diminished glycogen synthesis, insulin sensitivity, MAP kinase activity,
Y1158F	No effect on IRS-2 binding ⁵ Decreased IR internalization ¹³
Y1158, Y1162F, Y1163F	Deduction of insulin-activated auto-phosphorylation ¹⁶ Decreased IR internalization ¹³ Diminished glucose uptake
Δ 1312-1355	No significant influence on kinase activities ^{7, 10} No significant effect on IR internalization ¹³

⁴ (O'Neill *et al.*, 1994); ⁵(He *et al.*, 1996); ⁶(M. F. White *et al.*, 1988); ⁷(Backer, Schroeder, *et al.*, 1992); ⁸(Backer *et al.*, 1990); ⁹(Bonifacino & Traub, 2003); ¹⁰(Myers *et al.*, 1991); ¹¹(Backer, Shoelson, *et al.*, 1992); ¹²(Chaika *et al.*, 1999); ¹³(Carpentier *et al.*, 1993); ¹⁴(Cieniewicz *et al.*, 2016); ¹⁵(Y Xu *et al.*, 2011); ¹⁶(Ellis *et al.*, 1986).

I.3.3 Signaling transduction in adipocyte differentiation

Because of the important function in cellular signaling transduction, the RTKs regulate several essential cellular processes such as proliferation, differentiation, and tissue homeostasis (Lemmon & Schlessinger, 2010; F Liu & Zhuang, 2016). For instance, RTKs regulate the liver regeneration through the Erk1/2 signaling pathway (J Li *et al.*, 2011). In liver, the proliferation of hepatocytes (hepatocytes undergo two cell cycles) occur in the early 66 hours after partial hepatectomy (L Xu *et al.*, 2010). The RTK → SHC → GRB2/SOS → RAS → RAF pathway is found to promote the progression of G₁ phase during the proliferation, while the RTK → PLCγ → PKC → SRC/PYK2 → GRB2/SOS → RAS → RAF pathway participates in advancing the S phase and G₂/M phase checkpoint (J Li *et al.*, 2011). RTKs also regulate the differentiation of mesenchymal stem cells (MSCs) into neuronal cells (Tzeng *et al.*, 2015). Upon the stimulation of growth factors such as FGF-8 (fibroblast growth factor 8) and FGF-2 (fibroblast growth factor 2), RTKs induce activation of both the Erk1/2 signaling pathway and the PI3K/Akt signaling pathway in MSCs (Trzaska *et al.*, 2007). On the one hand, the phosphorylation of Erk in the former pathway phosphorylates the downstream protein GREB (cAMP response element-binding protein) and then leads to the up-regulation of TrkB (tyrosine receptor kinase B, the receptor of BDNF) and the secretion of BDNF (brain-derived neurotrophic factor) (Tzeng *et al.*, 2015). On the other hand, the activation of the later pathway plays a critical role in cell survival (Tzeng *et al.*, 2015). Therefore, the importance of RTKs on intracellular trafficking

and cellular signaling, and how these receptors regulate proliferation and differentiation will allow us to understand the critical role of these receptors in the process of adipogenesis.

Following up the functions of RTKs, the IR specifically activates two essential signaling pathways: the Erk1/2 signaling pathway and the PI3K/Akt signaling pathway (Figure 7). Through the two pathways, the activation of the IR regulates multiple biological processes: gene expression, protein translation, and cell growth via the Erk1/2 signaling pathway; and changes in metabolic function, such as glucose uptake, glycogen synthesis, and expression of necessary enzymes/proteins as acute metabolic response, through the PI3K/Akt signaling pathway (Taniguchi *et al.*, 2006; Jensen & De Meyts, 2009). On the one hand, Shc is recruited and activated through the pY972 on the juxtamembrane domain of the IR. Then Grb2 will be recruited via the interaction between its SH2 domain and tyrosine phosphorylated Shc (Jameson *et al.*, 2016). At last, SOS will be recruited and interacts with the two SH3 domains of Grb2 to form the Grb2-SOS complex, following the activation of RAS → RAF → MEK → Erk1/2 cascade (Zarich *et al.*, 2006). On the other hand, the phosphorylation on the Y972 recruits and activates both IRS-1 and IRS-2 whereas the Y1158, Y1162, and Y1163 on the activation loop of the IR are only responsible for IRS-2 recruitment and activation. Then PI3K will be recruited and activated based on its SH2 domains following IRS-1/2 activation (Youngren, 2007). The activated PI3K finally leads to activation of Akt, which ensures the IRS-1/2 → PI3K → Akt cascade.

Besides the effects on normal cell processes, the IR signaling pathways are essential for the differentiation of the pre-adipocytes in specific aspects. First, the signaling pathway is triggered by activation of the IR and regulates the differentiation, proliferation, and growth of the fat cells (adipocytes) by stimulating the formation and storage of lipids in both pre-adipocytes and adipocytes (Saltiel & Kahn, 2001). Second, the invagination of the IR complexes is activated through the RME, which regulates the IR distribution on the plasma membrane, insulin sensitivity, and density of the IR biological activities (Morcavallo *et al.*, 2014). Interestingly, the receptor signaling of IR complexes is not terminated after invagination, on the opposite, it can be magnified (Vieira *et al.*, 1996). Since the internalized IR complexes are still active and continue triggering the downstream signaling, the process of internalization is essential for the regulation of signal transduction (Sorkin & Von Zastrow, 2002). As is discussed above, there are two fates for these internalized vesicles: 1) to merge with lysosomes and be degraded; 2) to proceed to the recycling endosomes from where the IR is brought back to the cell surface to be used again (Marsh, 2001). The transport and sorting of early endosomes require the participation of Rab5 (Hunker *et al.*, 2006).

I.4 Regulating the function of Rab5

I.4.1 The small GTPase Rab5: small but essential

Rab5 is a Ras-like small GTPase (small GTP-binding protein) that not only shows a crucial regulatory role in controlling the RME of the IR but also plays a rate-limited role in early endosome fusion (Barbieri *et al.*, 2004). Like the other

GTPases, Rab5 has two forms: the active form and the inactive form. The active form is also the Rab5 GTP-bound form, while the inactive form is also the Rab5-GDP-bound form (Figure 8). The converting of the Rab5 GTP-bound form to the Rab5 GDP-bound form (inactivation of Rab5) is triggered by Rab5 GAPs while the activation of Rab5 is catalyzed by Rab5 GEFs (Figure 8).

Figure 8. Rab5 regulation.

As other GTPases, Rab5 has two forms: the active form and the inactive form. The active form is the Rab5 GTP-bound form while the inactive form is the Rab5 GDP-bound form. The converting of the GTP-bound form to the GDP-bound form (inactivation of Rab5) is triggered by GAPs while the converse activation process of Rab5 is catalyzed by GEFs (Lundquist, 2006). The ribbon diagrams of GTP/GDP-bound form of Rab5 are modified from Zhu et al. (Zhu et al., 2004). The switch I/II (SW (I)/(II)) regions are shown in red and blue, respectively. GppNHp (a nonhydrolyzable GTP analog), GDP, and metal ions are shown in brown sticks and dots, respectively. (PL: phosphate-binding loop; GAP: GTPase activating protein; GEF: guanine exchange factor; GppNHp: guanosine-5'(β,γ)-imidotriphosphate.)

As the primary Rab protein to function in early endosome dynamics and probably the one that has the best-characterized effectors in mammalian studies, Rab5 presents on the plasma membrane and endocytic vesicles, but mainly locates on sorting endosomes (Woodman, 2000). Rab5 is also a membrane-associated protein that undergoes a hydrophobic modification at the C-terminal (Woodman, 2000). The isoprenylation of C-terminal cysteines allow Rab5 to associate with the membrane of an organelle or transport vesicles in its GTP-bound form (Pechlivanis & Kuhlmann, 2006). When in the Rab5-GDP binding form, Rab5 can be maintained in a soluble state, transferred, and efficiently recruited in the cytosol by forming a cytosolic complex with guanine nucleotide dissociation inhibitor (GDI) (Woodman, 2000). The GDI displacement factors (GDFs), which present on the membrane of the endocytic vesicles or the early endosome, then catalyze the localization of Rab5 to these membrane compartments and mediate the replacement of GDI (Blumer *et al.*, 2013). A Rab5 GEF in the endosome membrane will then activate Rab5 by exchanging GDP to GTP (Alberts *et al.*, 2007). The activated Rab5 can activate PI3K, which synthesizes PI3P (phosphatidylinositol 3-phosphate). Together with PI3P, the activated Rab5 binds to a variety of Rab5 effectors to perform the biological effects of Rab5 on endosome fusion (Woodman, 2000). In addition, the activated Rab5 can recruit more Rab5 GEFs, thus creating a positive feedback loop to further enhancing Rab5 assembly. Interestingly, Rab5 is also reported to be required for the interaction between IRS-1 and p85 α and the activation of Akt upon insulin stimulation (Su *et al.*, 2006).

In summary, Rab5 activity is regulated by several Rab5 specific GEFs and GAPs, and more importantly, different Rab5 effectors are recruited by the GTP-bound Rab5 to modulate particular intracellular pathways (Table 3).

Table 3. Rab5 regulators and effectors.

	Regulator/Effector
Rab5 GEF	RIN1/2/3
	Rabex-5
	ALS2/Alsin
	RAP6/GAPex-5/RME-6
Rab5 GAP	RN-Tre
	RabGAP-5
Rab5 effectors	Rabaptin-5
	Vps34
	Rabenosyn-5
	EEA1
	APPL1/2
	PI3K
	Rabankyrin-5
	Huntingtin

I.4.2 Guanine nucleotide exchange factors, GTPase-activating proteins, and effectors of Rab5: working cooperatively

Rab5 GEFs, including Ras interference 1/2/3 (RIN1/2/3), Rabaptin-5 associated exchange factor for Rab5 (Rabex-5), amyotrophic lateral sclerosis 2 (ALS2/Alsin), GTPase activating protein and exchange factor for Rab5 (GAPex-5),

and Ras interference-like (RinI) are specialized by the highly conserved vacuolar protein sorting-associated protein 9 (VPS9) domain through which the inactivated Rab5 interacts with (Carney *et al.*, 2006). The VPS9 domain-containing proteins are widely conserved in eukaryotic cells of different species, such as Vps9p and Muk1p from *S. cerevisiae*, RME-6, RABX-5, and CE23604 from *Caenorhabditis elegans* (*C. elegans*) and at least nine proteins from the human beings (Carney *et al.*, 2006).

Functions of the Rab5 GEFs have been extensively studied in the past four decades. The RIN family is composed of RIN1, RIN2, and RIN3. They are multifunctional proteins that, besides the VPS9 domain, contain another three domains: the RA (Ras association) domain, the PR (proline rich) domain, and the SH2 domain. The RIN proteins share similar structure of all the domains but are distinguished by the containing of a different number of the PH domain, which has one/two/three repeats in RIN1, RIN2 and RIN3, respectively. The RIN proteins can be activated by the IR through the SH2 domain and activate Rab5 through the VPS9 domain upon IR activation. Activation of RIN proteins leads to the activation of Rab5, thus promotes the early endosome fusion (Barbieri *et al.*, 2004). The RA domain of the RIN proteins is responsible for the interaction with Ras, indicating RIN proteins to be Ras effectors that promote Rab5 activity. RIN1 is the most well-characterized protein in the RIN family, existing in both the cytoplasm and the plasma membrane (Colicelli *et al.*, 2012). Previous studies show that RIN1 promotes EGFR and TGF- β receptor internalization through Rab5 activation (Barbieri *et al.*, 2003; Colicelli *et al.*, 2012). The expression of RIN1-SH2 domain,

however, impairs the EGFR internalization without affecting cargo endocytic receptors, such as transferrin (Barbieri *et al.*, 2003). RIN1 also binds to 14-3-3 proteins through a sequence containing serine 351. The binding with 14-3-3 locates RIN1 to the cytoplasm and intracellular compartments whereas the mutation S351A shifts RIN1 localization to the plasma membrane (Y Wang *et al.*, 2002). RIN1:R94A mutant, which locates in the SH2 domain, showed a decreased interaction with EGFR upon EGFR activation (Barbieri *et al.*, 2003). The RIN1:Y561F mutant, which locates in the VPS9 domain of RIN1, showed defective function in activation of Rab5 and dramatic inhibition of Rab5 function (Galvis *et al.*, 2009). In addition, RIN1 has been found to be a breast tumor suppressor gene and undergoes a regulated expression in forebrain neurons and epithelial cells (Dzudzor *et al.*, 2010; Milstein *et al.*, 2007). RIN2 and RIN3 display same biochemical property as RIN1 that perform the guanine nucleotide exchange activity. However, both RIN2 and RIN3 can bind to amphiphysin II, whereas RIN1 cannot. Besides this distinct interaction, RIN1 is dominantly expressed in brain and pancreas, RIN2 has its highest expression in heart and kidney, and RIN3 exhibits higher expression in human mast cells (Carney *et al.*, 2006; Janson *et al.*, 2012). RIN3 also exhibits a unique intracellular localization as it locates on Rab5-positive vesicles in the cytoplasm but does not colocalize with the essential early endosome marker early endosome antigen 1 (EEA1) (Kajiho *et al.*, 2003).

The Rinl was recently identified as a Rab5 GEF with high similarity to the RIN proteins but without an RA domain (Kajiho *et al.*, 2012). It is ubiquitously expressed in all types of cells but with higher expression in lymphoid organs, such

as thymus and spleen, and specifically accumulates at neuromuscular synapses (Woller *et al.*, 2011). It colocalizes with Rab5 in actin-rich membrane ruffles, which indicates a novel role of Rin1 to recruit Rab5 to the cytoskeleton (Woller *et al.*, 2011). Interestingly, the Rin1 also activates Rab22 and even shows a higher catalytic activity for Rab22 compared to Rab5.

The Rabex-5 protein structure is identified to be made up of an N-terminal domain, a membrane binding domain, a helical bundle (HB) domain, a VPS9 domain, a coiled-coil (CC) domain, and a PR domain (Shin *et al.*, 2012). The Rabex-5 activates Rab5 and thus recruits Rabaptin-5 (a Rab5 effector), which then binds to the GTP-bound Rab5 and recruits Rabex-5 in a positive loop. Interestingly, the GTP exchange activity of Rabex-5 is much higher when in the Rabex-5-Rabaptin5 complex (Delprato *et al.*, 2004). Besides, the VPS9 domain of Rabex-5 alone shows a higher GTP exchange activity than the full-length Rabex-5 (Delprato *et al.*, 2004).

Alsin is a 183kDa protein that mainly expresses in brain. It is a multifunctional protein that contains a Ran GEF RCC1 (RCC1-like) domain, a tandem Dbl homology (DH) domain, a pleckstrin homology domain, several membrane occupation and recognition nexus (MORN) motifs, and a VPS9 domain (Carney *et al.*, 2006). Alsin exhibits colocalization with Rab5 and its effector EEA1 on the early endosomes in Hela cells (Otomo *et al.*, 2003). Overexpression of the wild-type Alsin and the constitutive active form of Alsin both results in enlarged endosomes in cultured neuronal cells through the activation of Rab5 (Otomo *et al.*, 2003). Besides, Alsin-deficient mice exhibit a disturbance in endosome

transportation of IGF-1R but unaltered endocytosis of transferrin and dextran (Devon *et al.*, 2006).

Rab5-activating protein 6 (RAP6) is the human homolog of GAPex-5, which is also known as receptor-mediated endocytosis protein 6 (RME-6) as the *C. elegans* homolog, contains an N-terminal RasGAP domain and a C-terminal VPS9 domain. Unlike the other Rab5 GEF proteins that colocalize with Rab5 on the early endosomes, RAP6 is primarily found on clathrin-coated pits (CCPs). *In vivo*, the expression of the dominant-negative form of Rab5 (Rab5-S34N, this mutant will be discussed later in other chapters) or small interfering RNA (siRNA)-mediated depletion of RAP6, reduced the uncoating efficiency of AP2 complex on the CCPs by increasing the steady-state levels of AP2 (Semerdjieva *et al.*, 2008).

Another group of proteins that regulate the Rab5 function are the GAPs, including the Rab5-specific GAP protein (RabGAP-5) and related to the N terminus of the oncogene (RN-tre), which solubilize Rab5 by inactivating Rab5 from the acceptor membrane (Figure 6). The rate of GTP hydrolysis mediated by GAPs is relatively high since the basal rate is intrinsically slow (~1 min) (Scheffzek *et al.*, 1998).

RN-tre localizes to Rab5-positive vesicles. Overexpression of RN-tre inhibits the Rab5-dependent internalization of transferrin and EGF but does not affect the membrane association of EEA1 (Haas *et al.*, 2005; Palamidessi *et al.*, 2013). Removal of RN-tre increases the level of active Rab5 and significantly improves the cell migration of mouse embryo fibroblasts *in vitro* (Palamidessi *et al.*, 2013).

The overexpression of RabGAP-5 results in a loss of Rab5 effector EEA1 from early endosomes and impaired endocytosis (Haas *et al.*, 2005). However, depletion of RabGAP-5 leads to similar phenotype as the expression of the constitutive active mutant of Rab5 (Rab5-S34N): level of endosome-localized EEA1 is increased, endosomes become larger, and trafficking of EGF is disrupted (Haas *et al.*, 2005).

In the processes of vesicle formation and delivery, Rab5 is also regulated by multiple Rab5 effectors. As one of the best studied Rab proteins to date, Rab5 has been found to interact with as many as 30 different effector proteins (Pfeffer, 2005). For instance, EEA1, an important Rab5 effector that binds to Rab5-GTP form, is the only effector that is necessarily required by minimal fusion and mediates endosome docking (Christoforidis *et al.*, 1999). Another Rab5 effector, Rabaptin-5, is initially identified as an GTP-bound Rab5 effector through a yeast 2-hybrid screen (Stenmark *et al.*, 1995). Rabaptin-5 is mostly found as a complex with Rabex-5 in the cell (Horiuchi *et al.*, 1997). Rabex-5 and Rabaptin-5 recruit each other in a positive loop upon Rab5 activation and thus keep the membrane Rab5-positive until the loop is interrupted by other recruited compartments (Kalin *et al.*, 2015). Deletion of the Rab5-binding domain of Rabaptin-5 results in giant endosomes as well as impaired endocytosis process (Kalin *et al.*, 2015).

In summary, the movement of intracellular vesicles mediated by RME is related with Rab5 and its effectors due to their roles in regulating vesicle formation and delivery.

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CHAPTER II

II. Role of Rab5 in the process of adipogenesis

II. 1 Introduction

As described in Chapter I, the *in vitro* differentiation of the mouse 3T3-L1 pre-adipocytes into mature adipocytes is an insulin-dependent ten-days process that can be divided into a mitotic clonal expansion (MCE) phase and a terminal differentiation phase (Tang & Lane, 2012). The pre-adipocytes undergo two cycles of mitosis during the MCE phase maintaining their fibroblast-like cell characteristics. However, the cells exhibit distinct morphological changes in the terminal differentiation phase. Besides the alterations in the cell shape and size, the most noticeable feature of the mature adipocytes is the formation and accumulation of a large number of lipid droplets in the cytosol (Suzuki *et al.*, 2011). The conversion from pre-adipocytes into adipocytes, along with the remarkable changes in cell morphology, is controlled by the insulin and IGF-1 signaling pathways and numerous transcription factors. Much of what is known about the adipogenic factors required for adipogenesis process is based on the *in vitro* studies of adipogenic cell lines, such as the mouse 3T3-L1 and 3T3-F442A cell lines. The essential transcription factors promoting the adipogenesis process are the peroxisome proliferator-activated receptor γ (PPAR γ) and the CCAAT/enhancer-binding protein (C/EBP) family (Rosen *et al.*, 2002). These transcription factors regulate the expression of most genes that define mature adipocytes, including the insulin-sensitive glucose transporter isoform 4 (GLUT4), which is primarily

expressed on the plasma membrane of adipocytes (James *et al.*, 1988; Tamori *et al.*, 2002). The expression level of GLUT4 and the number of GLUT4 existing on the plasma membrane is regulated through the insulin and IGF-1R signaling pathways (Bryant *et al.*, 2002; Czech & Buxton, 1993). Several Rab proteins are involved in the trafficking of GLUT4 by participating in the insulin signaling transduction process and the transportation of GLUT4 from intracellular GLUT4 storage vesicles (GSVs) to the plasma membrane (Sadacca *et al.*, 2013). In particular, three Rab proteins of the endosomal system (e.g., Rab5, Rab4, and Rab11) participate at different steps of the GLUT4 trafficking process.

Rab5 is a key regulatory molecule directing in the early endocytic pathway (Barbieri *et al.*, 2004; Barbieri *et al.*, 2000; Hoffenberg *et al.*, 2000). Rab5 is encoded as three differentially expressed isoforms, Rab5a, Rab5b, and Rab5c (Chen *et al.*, 2009). The mRNA expression profiling study shows distinct tissue distributions of the Rab5 isoforms (Gurkan *et al.*, 2005). A study shows that the suppression of Rab5a or Rab5b undermines the degradation of EGFR, whereas Rab5c shows limited effect (Chen *et al.*, 2009). Another study suggested the silencing of Rab5c, but not the other two isoforms, significantly reduced the focal adhesion foci of Hela cells, indicating a particular role of Rab5c in cell migration (Chen *et al.*, 2014). However, the functional redundancy of the isoforms is suggested as only the simultaneous knockdown of all three isoforms results in an inhibition of the insulin-dependent Akt activation, whereas individual knockdown of any single isoform does not affect Akt activation (Su *et al.*, 2006).

Mutational studies have shown that the Rab5:S34N, a Rab5 GTP-binding defective mutant, inhibits transferrin and EGF endocytosis displaying a dominant-negative effect; whereas the Rab5:Q79L, a mutant with reduced GTPase activity, behaves as a constitutively active Rab5 mutant stimulating internalization of several ligands, such as EGF and insulin (Dinneen & Ceresa, 2004; Hunker *et al.*, 2006; Jozic *et al.*, 2011; Jozic *et al.*, 2012; Mendoza *et al.*, 2013). The Q79 residue of Rab5 locates on the GTPase domain (residues 15-184) of Rab5. Structural study of Rab5 reveals that each GTPase domain of Rab5 binds to one GTP and one Mg²⁺ ion and contains a central six-strand β -sheet (β 1- β 6) flanked by five α -helices (α 1- α 5) (Terzyan *et al.*, 2003). Two switch regions, switch I (residues 44-64) and switch II (residues 76-94) undergo structural changes between the GTP-bound and GDP-bound forms (G Zhu *et al.*, 2004). Several residues on Rab5 have been reported to be responsible for the interaction and recruitment of specific Rab5 effectors. For instance, Y57A, W74A, Y82A, and Y89A based on the Rab5:Q79L mutant showed significant decreased binding affinity with Rabaptin-5, whereas K116E and R120E based on the Rab5:Q79L mutant had no effect on Rab5-Rabaptin-5 binding (G Zhu *et al.*, 2004). Moreover, Y57A, W74A, Y82A, and Y89A are also found to be effector binding epitopes for EEA1 and Rabenosyn-5 based on structural studies (Mishra *et al.*, 2010).

Like the other small GTPases, the Rab5 cycle is regulated by several Rab5 GEFs (e.g., RIN1, Rabex-5, and Rin1) and Rab5 GAPs (e.g., RN-tre and RabGap-5). Rab5 in its active form (the GTP-bound form) recruits several downstream effectors onto the endosome membrane to support the endosomal fusion in the

early endocytic pathway and the interaction of endosomes with microtubule networks (Balaji *et al.*, 2012; Sandri *et al.*, 2012). Rab5 is also implicated in other cellular processes such as receptor cell signaling and phagosome maturation (Mustafi *et al.*, 2013). Recently, Rab5 is also proved to participate in several cellular functions, including endoplasmic reticulum (ER) reorganization, nuclear Lamin disassembly, spindle assembly and stability during mitosis (Audhya *et al.*, 2007; Capalbo *et al.*, 2011; Das *et al.*, 2014). Furthermore, up-regulation of the Rab5-GTP level upon addition of EGF requires the participation of one or more Rab5 GEFs (Hunker *et al.*, 2006; Jozic *et al.*, 2011; Jozic *et al.*, 2012). Interesting, the addition of insulin to 3T3-L1 pre-adipocytes, but not the NIH-3T3 or the HepG2 cell lines, induced inactivation of Rab5 (Huang *et al.*, 2001; Tessneer *et al.*, 2014). Thus, evidence regarding the regulation of Rab5 activity during adipogenesis process and the understanding of the cellular mechanisms by which Rab5 modulates the differentiation of 3T3-L1 pre-adipocytes are lacking.

Besides Rab5, several Rab proteins (e.g., Rab4, Rab7, Rab22, and Rab31) also have their particular roles in the endocytosis process. *In vitro* study of 3T3-L1 cells reveals an increased cytosolic Rab4 level upon insulin stimulation (Shisheva & Czech, 1997). Rab4 is also proved to play a role in determining directional vesicle mobility (Potokar *et al.*, 2012). Similar as Rab5, the overexpression of the dominant-negative mutant of Rab4 (Rab4:Q67L) results in enlarged endosomes and diminished vesicle mobility in terms of vesicle track length (the total length that the vesicle traveled during analysis), maximal displacement (maximal distance between two positions in the track that the vesicle traveled during analysis), and

speed (Potokar *et al.*, 2012). Consistently, the overexpression of the dominant-positive mutant of Rab4 (Rab4:S22N), leads to dysfunctional vesicle directionality (Potokar *et al.*, 2012). If Rab5 is the key for homotypical early endosome fusion, then Rab7, which controls the net movement of late endocytic elements, is the star to govern the early to late endosome fusion (Lebrand *et al.*, 2002). It is reported that Rab7 colocalizes with the insulin-responsive GLUT4-containing intracellular compartments, but its localization is not affected by the stimulation of insulin (Millar *et al.*, 1999). The deletion of Rab7 in Hela cells does not affect the trafficking of the EGF-EGFR complex through the early endosome to the late endosome upon EGF stimulation. However, the EGF-EGFR complex is sequestered in the late endosomes indicating a regulatory role of Rab7 in the receptor trafficking (Vanlandingham & Ceresa, 2009). Rab11 is a master regulator to mediate the transportation of intracellular vesicles towards the cell surface in the exocytosis process (Takahashi *et al.*, 2012). Depletion of Rab11 results in tubulated recycling endosomes as well as the accumulation of recycling compartments containing endocytosed transferrin and transferrin receptor beneath the plasma membrane (Takahashi *et al.*, 2012). Rab22 is from the same subfamily of small GTPases as Rab5 and thus displays high sequence homology to Rab5 (Kauppi *et al.*, 2002). A previous study showed that Rab22 even perform a higher affinity to the Rab5 GEF, Rabex-5, interacted with Rabex-5 to recruit the Rab5 effector, EEA1, and promoted early endosome fusion (HP Zhu *et al.*, 2009). The knockdown of Rab22 abrogates the membrane targeting of Rabex-5 in the body hamster kidney (BHK)-21 cells as well as the NGF-induced TrkA endocytosis in the PC12 cells (Wang *et*

al., 2011; HP Zhu *et al.*, 2009). Rab31 is also a Rab5 subfamily GTPase that can be activated by the Rab5 GEF, RAP6, and perform its function in *trans*-Golgi Network-to-endosome trafficking (Lodhi *et al.*, 2007). The overexpression of Rab31 impairs the GLUT4 translocation from the intracellular compartments to the cell surface whereas the knockdown of Rab31 potentiates both GLUT4 translocation and glucose uptake upon insulin stimulation (Lodhi *et al.*, 2007).

Because of the essential role of Rab proteins in the regulation of endocytosis as well as in trafficking of glucose, I hypothesized that Rab5 and other Rab proteins participate in the 3T3-L1 pre-adipocytes adipogenesis process positively.

II.2 Specific aims and research design

II.2.1 Specific aim 1: To examine the key determinants of Rab5 that are required in adipogenesis.

Rab5 is a small GTPase required for several cellular processes, including proliferation, migration, and invasion. It functions as a molecular switch as it cycles between its GTP-bound form (the active form) and GDP-bound form (the inactive form). The switch of Rab5 is controlled by guanine nucleotide exchange factors (GEFs), which activate Rab5 by increasing the nucleotide status of Rab5 in the GTP-bound form, and also by GTPase activating proteins (GAPs), which inactivate Rab5 by accelerating the hydrolysis of GTP to GDP. Preliminary observations indicated that the expression of Rab5-Wild-type (WT) affected adipogenesis. Therefore, I determined

- 1.a the effect of the Rab5 isoforms (Rab5A, Rab5B, and Rab5C) on adipogenesis.
- 1.b the nucleotide status of Rab5 during adipogenesis
- 1.c the effect of the deletion of the N-terminal or C-terminal of Rab5 on adipogenesis.
- 1.d the effect of point mutants in the effector domain of Rab5 on adipogenesis.
- 1.e the particular role of Rab5 in the differentiation process in comparison with other Rab5 family proteins.

II.2.2 Research design 1

Rab5 isoforms, and several point and deletion mutants of Rab5, as well as several selected Rab proteins, were inserted into the pMX-puro vector at the EcoRI and NotI restriction sites. Then the plasmid DNA were purified via the plasmid purification assay. The purified DNA was transfected into the Plat-A cells using Lipofectamine® 2000 reagent as specified by Invitrogen. After 48 hours, virus suspension from the Plat-A cells was applied to the 3T3-L1 mouse pre-adipocyte cell line for the expression of specific proteins. The transfected cells were then selected with puromycin. siRNA for the Rab5 isoforms were designed and synthesized by Ambion. The purchased specific siRNA was induced into the 3T3-L1 cells by Lipofectamine® 2000 reagent as specified by Invitrogen. The siRNA-transfected or the selected pMX-puro vector-transfected 3T3-L1 cells were utilized for several biological assays, including cell cycle testing, lipid accumulation testing (ORO assay, only for differentiated cells), microscopy, and western blotting.

Cell lysates of the 3T3-L1 cells that have been transfected with GFP, Rab5:WT, Rab5:Q79L, and Rab5:S34N were incubated with GST-labeled EEA1. The bonded proteins were eluted and be used for immunoprecipitation analysis.

II.3 Material and Methods

II.3.1 Materials

The 3T3-L1 pre-adipocyte cell line was obtained from the Zen-Bio, Inc. (NC, USA). The Platinum A (Plat-A) retroviral packaging cell line and the pMX-puro retroviral vector were purchased from Cell Biolabs, Inc. (San Diego, CA, USA). Primary antibodies, including the insulin receptor (IR), GLUT4, Akt1, p-Akt (Ser473), PPAR γ , C/EBP α , Erk1/2, p-Erk1/2, FABP4, FAS, ACC, GAPDH, and Rab5, and secondary antibodies, including the anti-Rabbit IgG and anti-Mouse IgG, were purchased from the Cell Signaling Technology (Boston, MA, USA). Fetal bovine serum (FBS) was purchased from the RMBIO, Inc. (Missoula, MT, USA). Other reagents and supplies, if not specifically described, were purchased from Thermo Fisher Scientific (Weston, FL, USA).

II.3.2 cDNA Construction.

Complementary DNAs (cDNA) of green fluorescent protein (GFP), Rab5 isoforms, Rab4:wild-type (Rab4:WT), Rab7:WT, Rab11:WT, Rab22:WT, Rab31:WT, GFP-Rab5:WT, GFP-Rab5:S34N, and GFP-Rab5:Q79L were cloned into the pMX-puro vector at the EcoRI and NotI restriction sites (Onishi *et al.*, 1998). GFP-Rab5:S34N, GFP-Rab5:Q79L, Rab5:S34N, Rab5:Q79L, Rab4:S22N,

Rab4:Q67L, Rab7:S22N, Rab7:Q67L, Rab11:S22N, Rab11:Q70L, Rab22:S19N, Rab11:Q64L, Rab31:S19N, and Rab31:Q64L on the pMX-puro vector were made via the Q5[®] Site-Directed Mutagenesis Kit purchased from New England Biolabs, Inc. (NEB, Ipswich, MA, USA). Rab5:Q79L- Δ C4, Rab5:Q79L- Δ N19, Rab5:S34N- Δ C4, Rab5:S34N- Δ N19, and GFP-Rab5:Q79L double mutants (GFP-Rab5:Q79L-W57A, Rab5:Q79L-W74A, Rab5:Q79L-Y82A, Rab5:Q79L-Y89A, Rab5:Q79L-K116E, and Rab5:Q79L-R120E) on the pMX-puro vector were purchased from GenScript, Inc. (Piscataway, NJ, USA).

II.3.3 Site-directed mutagenesis.

5' and 3' primers for specific mutant were designed via the online NEB primer design software, NEBaseChanger[™], and obtained from Sigma-Aldrich, Inc. (Raleigh, NC, USA). The site-directed mutagenesis process was done following the protocol of the Q5[®] Site-Directed Mutagenesis Kit. Briefly, 0.5 μ M of 5' and 3' primers were mixed with 25 ng of template vector DNA in the Q5 Hot Start High-Fidelity 2X Master Mix reagent. 25 cycles of PCR were performed followed by the kinase, ligase, and DpnI treatment for 5 minutes. The treated PCR mixture was then used for bacteria transformation, which will be described later.

II.3.4 Bacteria transformation

The cDNA-containing vectors (either purchased or cloned) and the treated PCR mixture generated via the site-directed mutagenesis were transformed into the 5-alpha Competent *E. coli* purchased from NEB via the transformation protocol

described on the company website. Briefly, the competent cells were thawed on ice for 10 minutes. Then 100 ng of cDNA-containing vectors or 5 µl of the treated PCR mixture was mixed with 50 µl of the competent cells in an Eppendorf tube and incubated on ice for 30 minutes followed by a 30 seconds heat shock at 42°C. Then the Eppendorf tube was placed on ice for 5 minutes before mixing with 950 µl of SOC media provided by the kit. After a one-hour incubation at 37°C with shaking (250 rpm), the transformed competent cells were spread on Lysogeny broth (LB) agar selection plates and incubated overnight at 37°C.

II.3.5 Vector DNA purification assay.

Colonies grown on the LB agar selection plates or the cDNA constructs were grown in the LB media overnight at 37°C. Then vectors containing specific cDNA constructs were collected according to the QIAprep Spin Miniprep Kit or Qiagen Plasmid Plus kits obtained from Qiagen Sciences, Inc. (Germantown, MD, USA). The optical density (OD) of vector DNA was tested and analyzed to ensure a high concentration and purity of DNA that can be used for mammalian cell transfection and yeast transformation.

II.3.6 Generation of stable cell lines

The cDNA containing vector was transfected into the Plat-A (80%~90% confluence) monolayer using Lipofectamine® 2000 Transfection Reagent. The Plat-A cells were grown in the Plat-A growth media (1% penicillin/streptomycin (P/S), 1 µg/mL puromycin, 10 µg/mL blasticidin, and 10% FBS in Dulbecco's

modified Eagle's medium (DMEM)) and incubated under a humid atmosphere containing 5% CO₂ under 37°C before and after transfection. 48 hours after transfection, the supernatant of media from the transfected Plat-A cells was filtered with 0.2 nm filter and applied to 90% confluent 3T3-L1 cells grown in the 3T3-L1 growth media (1% P/S, 1% L-glutamine, and 10% FBS in DMEM). After another 24-hour incubation, transfected 3T3-L1 cells were selected with the 3T3-L1 selection media (2µg/mL puromycin in 3T3-L1 growth media).

II.3.7 Cell culture and 3T3-L1 cell differentiation.

Non-transfected 3T3-L1 cells were grown to 100% confluence in the 3T3-L1 growth media and incubated in a humid atmosphere containing 5% CO₂ under 37°C. The differentiation of the 3T3-L1 pre-adipocytes took a total of 10 days. The differentiation process was triggered by treatment of the 3T3-L1 differentiation media (10 µg/ml insulin, 1 µg/ml DEX and 1µg/ml IBMX in the 3T3-L1 growth media for day 1 to day 4 and 10 µg/ml insulin in the 3T3-L1 growth media for day 5 to day 10). Media was changed every 48 hours. The pMX-puro cDNA-containing vector-transfected 3T3-L1 cells were grown in the same conditions as the non-transfected 3T3-L1 cells except with an addition of 2µg/mL puromycin in all types of media.

II.3.8 Oil Red O (ORO) staining and quantification.

On the tenth day of differentiation, the 3T3-L1 cells were washed and fixed with 10% formalin in phosphate buffer saline (PBS) for 5 minutes and 1 hour at room temperature, respectively. The ORO stock solution (0.5 g of ORO in 100 ml

of isopropanol) was diluted freshly with deionized (DI) water to make the ORO working solution (60% of stock solution with 40% of DI water). The working solution was filtered with a 0.2 nm filter before applying to the fixed 3T3-L1 cells. Then the 3T3-L1 cells were stained with the filtered ORO working solution for 15 minutes at room temperature. Excessive ORO working solution was removed and the stained 3T3-L1 cells were washed with DI water. At last, 100% of isopropanol was used to elute the ORO from fixed cells by incubating the cells in a 37°C water bath for 10 minutes. The quantification of lipids was done by measuring OD of eluted ORO solution at 540 nm wavelength.

II.3.9 Bright-field microscopy, confocal microscopy, and live-cell microscopy

For bright-field and confocal microscopy, the normal 3T3-L1 cells and the transfected 3T3-L1 cells were seeded on glass coverslips at 1.0×10^5 per 3.8 cm² well. The cells were incubated with different types of media and treatments as required. After the ORO staining (as described above), the cells were observed and taken image of under an inverted microscope at a 20X magnification objective lens via the AmScopeX software for bright-field images. Or the cells were mounted with DAPI and observed under the Olympus BX61 confocal microscope.

For live-cell microscopy, the normal/transfected 3T3-L1 cells were seeded in the μ -Slide 4 Well chamber purchased from ibidi, Inc. (Fitchburg, WI, USA) with required media and treatments. Then live-cell images were taken under the Delta Vision Elite microscope.

II.3.10 Lysate preparation, protein quantification, and Western blotting

II.3.10.1 Lysate preparation

To prepare whole cell lysates, 3T3-L1 cells were collected in ice-cold fresh lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium deoxycholate (w/v), 0.1% SDS, 1 mM Na_3VO_4 , 10 mM NaF, 2 mM phenylmethane sulfonyl fluoride (PMSF), and protease/phosphatase inhibitors purchased from Sigma-Aldrich, Inc.). 3T3-L1 cell lysates were then mixed with the 4X loading dye and heat-shocked at 100°C for 10 minutes. Supernatant were collected after centrifugation (2 minutes in 13,000 rpm) for western blotting. To prepare the whole yeast lysates, the yeast cells were first collected by centrifugation (5 minutes in 5000 rpm). After discarding the supernatant, 3 times of freeze-thaw cycles (3 minutes in -20°C ethanol then 3 minutes in 37°C water bath) were applied. The cells were then mixed with the fresh lysis buffer with addition of 5 mM of DTT for 30 minutes.

II.3.10.2 Protein quantification – the Pierce BCA Protein Assay

Protein contents of the cell lysates were tested using the Pierce BCA Protein Assay Kit. Briefly, the working solution was made freshly by mixing the reagent A and the reagent B in a ratio of 1:20. Gradient volumes of the standard protein and same volume of sample proteins (preparation has been described above) were mixed thoroughly and incubated at 30°C for 30 minutes. The ODs of standards and samples were measured under an absorbance of 562 nm

wavelength. Then the concentrations of sample proteins were calculated based on the standard curve.

II.3.10.3 Western blotting

The tested cell lysates were mixed with the 4X loading dye and heat-shocked in 95°C for 10 minutes. Supernatant was collected after centrifugation (2 minutes in 13,000rpm). Same amount of cell lysate proteins (based on the BCA assay) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked overnight with specific primary antibodies at 4°C in BSA-TBS-t (20 mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween 20, and 5% bovine serum albumin (BSA)). After washing of the primary antibodies, specific secondary antibodies were applied and washed. Visualization of results was achieved by exposing. To determine relative protein amounts, three representative exposures for each sample were quantified using the Fiji software (National Institutes of Health).

II.3.11 siRNA sequences and transfection.

RNA interference (RNAi) directed against:

mouse Rab5A [5'-AAGCACAGTCCTATGCAGATG-3'],

mouse Rab5B [5'-AATCCGTGTGTTTAGATGACA-3'], or

mouse Rab5C [5'-AAGCAGCCATTGTGGTCTATG-3'] were designed and synthesized by Ambion (Austin, TX). A scrambled siRNA sequence was designed as a control [5'-CACCUAUCCGUGGUUCA-3']. Prior to siRNA sequence

transfection, cells were plated in growth medium without antibiotics at 70-80% confluence. siRNA sequence (20 nM final concentration) transfection was performed using Lipofectamine™ 2000 as specified by Invitrogen (Invitrogen, Carlsbad, CA). After transfection, cells were used for the indicated assay (e.g., for immunoblotting, differentiation or pull-down assays).

II.3.12 Activation of Rab5 assay.

Cells were lysed using a buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM DTT, 5 mM MgCl₂, 5% glycerol and 1% Triton-X-100, supplemented with 1 mM PMSF. Lysates were incubated with 100 mL glutathione beads containing 10 µg of GST-EEA1, followed by rocking for 1 hour at 4°C. After incubation, beads were washed three times with PBS. The pull-downs were subjected to SDS-PAGE and analyzed by immunoblotting using Rab5 antibodies. Relative levels of Rab5-GTP were determined using the ratio of Rab5- to total-Rab5.

II.3.13 Statistical analysis

All experiments presented were repeated a minimum of three times. Normal distributed data were represented in mean ± standard deviation (S.E.M.). The difference between groups were determined by the Student t-test. Tests were considered statistically significant if *p*-value were less than 0.05.

II.4 Results

II.4.1 Nucleotide status of Rab5 is critical for the differentiation of 3T3-L1 pre-adipocytes

Rab5 is known to regulate membrane fusion during vesicular trafficking where the Rab5:Q79L mutant, which has preferential affinity for GTP showed a dominant-positive effect and promotes early endosome fusion (Barbieri *et al.*, 1996). In contrast, the Rab5:S34N mutant, which has preferential affinity for GDP and shows a dominant-negative effect, induced the accumulation of very small endocytic profile and inhibited transferrin endocytosis (Stenmark *et al.*, 1994).

II.4.1.1 The induction of pMX-puro retroviral vector expression-system did not alter the differentiation process

To access whether Rab5 plays a regulatory role in the 3T3-L1 pre-adipocytes differentiation, I used the pMX-puro retroviral vector expression-system to induce the expression of a specific protein. The retroviral system is designed to express a specific exogenous protein in mammalian cells permanently. To establish that the transfection system will not affect the normal differentiation process, I induced the green fluorescent protein (GFP) into the 3T3-L1 cells as a control. In Figure 9.A, the overexpression of GFP alone did not affect the accumulation of lipids as compared with the un-transfected 3T3-L1 cells. As expected, the expression level of GAPDH, which served as the whole cell lysate loading control, was similar in both GFP-transfected and un-transfected cells before (day 1) and after (day 9) differentiation, respectively (Figure 9.B). Both cells

expressed similar amount of PPAR γ at day 9, suggesting that the usage of the pMX-puro retroviral vector expression-system did not significantly alter the differentiation process of the 3T3-L1 pre-adipocytes. Bright-field images of ORO stained cells were taken at day 1 and day 9 after differentiation (Figure 9.C).

Figure 9. Differentiation of un-transfected and GFP-overexpressing 3T3-L1 pre-adipocytes.

The un-transfected (3T3-L1) or GFP-overexpressing (GFP) 3T3-L1 pre-adipocytes were differentiated into adipocytes in the absence or in the presence of induction media, and the accumulation of lipid droplets was measured by the incorporation of Oil Red O as described in Material and Methods. A. Relative lipid content of 3T3-L1 and GFP 3T3-L1 cells. Results were represented as relative lipid contents. Data represent the mean $\pm$ S.E.M. of three independent experiments. B. Representative Western blot of the content of GFP, PPAR γ , and GAPDH proteins. C. Bright-field images of ORO stained day 1 and day 9 cells under 20X objective lens. ORO stained lipid droplets were shown as red color under the microscope.

II.4.1.2 The overexpression or deletion of individual Rab5 isoforms inhibited the differentiation of 3T3-L1 pre-adipocytes

For the purpose of understanding the role of different Rab5 isoforms during adipogenesis, individual overexpression of the Rab5 isoforms (Rab5A, Rab5B, and Rab5C) was prepared. As shown in Figure 10.A, all the Rab5 isoforms, Rab5A, Rab5B, and Rab5C, showed a statistically inhibitory effect on the lipids accumulation. Specifically, the overexpression of Rab5A inhibited the accumulation of lipids by $32\pm6\%$, while Rab5B and Rab5C inhibited the accumulation by $25\pm6\%$ and $21\pm5\%$, respectively. Then the depletion effect of each Rab5 isoforms was done via the RNA interference to individually or simultaneously knockdown the Rab5 isoforms. The accumulation of lipids in differentiated 3T3-L1 cells was examined under the knockdown of Rab5 isoforms by inducing specific siRNA. According to Figure 10.B, depletion of Rab5 was accompanied by a reduction of the accumulation of lipids. Importantly, individual removal of Rab5A isoform produced a small but statistically significant ($24\pm6\%$) inhibition on the accumulation of lipids as compared with scrambled siRNA-induced and siRNA-uninduced cells. Rab5B and Rab5C siRNA were also able to block the accumulation of lipids, though not as noticeable as Rab5A, by $14\pm2\%$ and $16\pm4\%$, respectively, as compared with scrambled siRNA and non-siRNA induced cells. Remarkably, combined depletion of all the Rab5 isoforms strongly inhibited ($84\pm5\%$) the accumulation of ORO (Figure 10.B).

Figure 10. Selective effect of Rab5 isoforms on the differentiation of 3T3-L1 pre-adipocytes.

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). A. Relative lipid content of cells expressing GFP or Rab isoforms. GFP or Rab5 isoforms-transfected 3T3-L1 cells were differentiated into adipocytes in the presence of induction media. B. Relative lipid content of differentiated 3T3-L1 cells with depletion of the Rab5 isoforms. Non-transfected cells (Ctl), or cells transfected with scramble RNAi (Scr), or individually RNAi against the Rab5 isoforms (siRab5a/b/c), or RNAi's against all Rab5 isoforms (siRab5abc) were differentiated as described in Material and Methods.

Since the Rab5A exhibited the most remarkable results in lipid formation during adipogenesis of the 3T3-L1 pre-adipocytes, the rest of the experiments were designed based on the Rab5A isoform. And in the rest of this section, Rab5 represents the Rab5A isoform if not specifically stated.

II.4.1.3 The GTP-bound Rab5 decreased during adipogenesis

As described above, Rab5 functions as a molecular switch that cycles between the active GTP-bound form and the inactive GDP-bound form. The overexpression and depletion of the three Rab5 isoforms individually exhibited inhibitory role in the adipogenesis process of 3T3-L1 pre-adipocytes (Figure 10). Thus, it is hypothesized that the nucleotide states of Rab5 may change during the differentiation process and the states of Rab5 may play a regulatory role in governing the adipogenesis process of 3T3-L1 cells.

In order to detect the endogenous nucleotide states of Rab5 during the differentiation of 3T3-L1 pre-adipocytes, the 3T3-L1 cells overexpressing GFP were differentiated into adipocytes in the absence (as the negative control) or in the presence (as the positive control) of induction media. Endogenous GTP-bound activated Rab5 was analyzed by Rab5 GST pull-down assay followed by immunoblotting at specific days during the differentiation. In Figure 11, along with the progression of adipogenic differentiation, the expression level of the adipocyte-specific transcription factor, PPAR γ , gradually elevated (Figure 11.A&B). In contrast, the relative Rab5-GTP level gradually decreased over time during the differentiation process (Figure 11.C&D).

Figure 11. GTP levels of endogenous Rab5 in comparison with expression of PPAR γ during the differentiation of 3T3-L1 pre-adipocytes.

3T3-L1 pre-adipocytes overexpressing GFP were differentiated into adipocytes in presence of induction media, and the endogenous GTP-bound activated Rab5 was analyzed by GST pull-down assay followed by immunoblotting at specific day of the differentiation as described in Material and Methods. A. Relative PPAR γ expression levels in comparison with GAPDH during differentiation. Data represent the mean $\pm$ S.E.M. of three independent experiments. (* $P < 0.05$ by Student's t-test). B. Representative Western blot of the content of PPAR γ and GAPDH. C. Relative Rab5 GTP-bound levels as compared with total Rab5 loaded. Data represent the mean $\pm$ S.E.M. of three independent experiments. (* $P < 0.05$ by Student's t-test). D. Representative immunoblots of samples probed with anti-Rab5 antibodies for total Rab5 (T-Rab5) and active Rab5 (GTP-Rab5).

II.4.1.4 Increase the Rab5-GTP level hampered the differentiation of 3T3-L1 pre-adipocytes

In order to address the role of Rab5 in the differentiation of 3T3-L1 pre-adipocytes, cell lines overexpressing GFP, Rab5:wild-type (WT), the GTP-bound defective Rab5 mutant (Rab5:S34N), or the GTPase-defective, constitutively activated Rab5 mutant (Rab5:Q79L) were prepared, and the accumulation of lipids was measured via the oil red O (ORO) assay. To observe the localization of Rab5 and the two Rab5 mutants, the GFP conjugated Rab5:WT, Rab5:S34N, and Rab5:Q79L were transfected into the 3T3-L1 cells. Then the day 1 and day 9 cells were fixed for confocal microscopy and observed under the Olympus BX61 confocal microscope (Figure 13). As shown in Figure 12, the overexpression of Rab5:Q79L mutant decreased the accumulation of lipids in 3T3-L1 pre-adipocytes at day 9 of differentiation. Moreover, Rab5:WT decreased the accumulation of lipids in less extent as compared with the Rab5:Q79L mutant but is statistically significant in comparison with the GFP cells (Figure 12.A). Surprisingly, the expression of Rab5:S34N mutant stimulated this differentiation process as the lipid content increased by about 28% in comparison with the GFP cells (Figure 12). The observation suggests that Rab5 inactivation improves the differentiation of 3T3-L1 pre-adipocytes, whereas constitutively activating Rab5 resulted in decreased lipid accumulation.

Figure 12. Selective effect of Rab5 constructs on the differentiation of 3T3-L1 pre-adipocytes.

3T3-L1 pre-adipocytes expressing GFP or Rab5 constructs (Rab5:WT, Rab5:S34N, and Rab5:Q79L) were differentiated as described in Material and Methods. A. Relative lipid content of 3T3-L1 cells overexpressing GFP, Rab5:WT, Rab5:Q79L, and Rab5:S34N. 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). B. Representative Western blot of the content of GFP, Rab5, and GAPDH proteins.

Adipocyte differentiation can be also monitored by formation of intracellular lipid droplets (Qi *et al.*, 2017). Thus, with the overexpression of GFP-conjugated Rab5 and Rab5 mutants, combining with the ORO staining assay, morphological experiments were carried out to visualize the lipid droplets and the localization of Rab5 or Rab5 mutants. Under the microscope, GFP and GFP-conjugated constructs were visualized as green, ORO stained lipid droplets were observed as red, whereas nucleus were stained with DAPI, which gave a color of blue under the microscope. As described above, 3T3-L1 pre-adipocytes expressing GFP and Rab5 constructs were cultivated and induced to differentiate into adipocytes with induction media. At day 9, Oil Red O staining showed an abundant red round structures in GFP- and Rab5:S34N-overexpressed 3T3-L1 cells, which suggested a significant lipid accumulation in the GFP-control and the Rab5:S34N mutant cells. However, lipid droplets were not observed in untreated non-differentiated day 1 cells (images in the left column of Figure 13). More importantly, formation of lipid droplets was inhibited in the Rab5:Q79L mutant cells (Figure 13). In cells overexpressing the Rab5:WT, lipid droplets were observed but in less amount in comparison with the GFP-control cells (Figure 13). These observations were further supported with the quantitative measurement of lipid content (Figure 12). In addition, the overexpression of Rab5:Q79L mutant significantly inhibited adipogenic morphology (e.g., the transition from a fibroblast-like shape to an increasingly rounded-up appearance with an accumulation of cytoplasmic lipid droplets), which can be confirmed by comparing the GFP-expressing cells with the Rab5:Q79L-expressing cells at day 9 of differentiation.

Figure 13. Confocal images of 3T3-L1 cells overexpressing GFP and GFP-conjugated Rab5 constructs.

GFP, lipid droplets, and nucleus were represented in green, red, and blue, respectively. Morphological changes of 3T3-L1 pre-adipocytes were monitored by a Olympus BX61 and photographed before (day 1) after 9 days from the onset of differentiation.

Figure 14. Effect of Rab5 constructs on the expression of PPAR γ and CEB/ α . 3T3-L1 pre-adipocytes overexpressing GFP or Rab5 constructs were differentiated as described in Material and Methods, and the endogenous levels of PPAR γ , CEB/ α and GAPDH proteins were analyzed with specific antibodies by immunoblotting. Data represent the mean $\pm$ S.E.M. of three independent experiments. (* P <0.05 by Student's t-test).

Next, the 3T3-L1 pre-adipocytes overexpressing GFP, Rab5:WT and the Rab5 mutants were differentiated and then harvested for immunoblotting analysis with specific antibodies. With the overexpression of Rab5:S34N mutant, but not the Rab5:Q79L mutant, the protein expression of the adipogenesis-associated transcription factors, PPAR γ and C/EBP α , were up-regulated as compared with the GFP-overexpressing cells (Figure 14). The Rab5:WT affected the expression of the two transcription factors in less extent in comparison with the Rab5:Q79L mutant. Thus, these observations suggest that the nucleotide status of Rab5 is critical for the differentiation process of 3T3-L1 pre-adipocytes. The effect of Rab5 nucleotide status on adipogenesis relies, at least partially, on its regulatory role towards the two adipogenic transcription factors, PPAR γ and C/EBP α .

II.4.2 The determinants of Rab5 in promoting adipogenesis.

In the previous section, Rab5:Q79L, the dominant-positive mutant of Rab5, has demonstrated an inhibitory role in adipogenesis process. Since specific residues, especially the Y57A, W74A, Y82A, and Y89A residues on the Rab5 GTPase domain affect the binding affinity of Rab5 with Rab5 effectors (e.g. Rabaptin-5, Ranenosyn-5, and EEA1), several additional mutants (Y57A, W74A, Y82A, Y89A, K116E, and R120E) were made based on the Rab5:Q79L mutant. Then the Rab5:Q79L double mutants were transfected into the 3T3-L1 cells through the pMX-puro vector system, respectively. Surprisingly, the Rab5:Q79L-K116E double mutant, not the other mutants that showed positive role in effector binding, advanced the lipid formation of differentiated 3T3-L1 cells, whereas the

other double mutants exhibited similar inhibitory role in adipogenesis as the Rab5:Q79L single mutant (Figure 15).

Figure 15. Selective effects of Rab5:Q79L double mutants on the differentiation of 3T3-L1 pre-adipocytes.

3T3-L1 pre-adipocytes overexpressing GFP, Rab5:Q79L, or Rab5:Q79L double mutants were differentiated into adipocytes in the presence of induction media, and the accumulation of lipid droplets was measured by the incorporation of ORO as described in Material and Methods. A. Relative lipid content of differentiated 3T3-L1 cells overexpression GFP, Rab5:Q79L mutant, and Rab5:Q79L double mutants. Data represent the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$ by Student's t-test. B. Representative Western blot of the content of GFP, Rab5 constructs, and GAPDH proteins.

Figure 16. Bright-field images of 3T3-L1 cells overexpressing GFP, Rab5:Q79L, or Rab5:Q79L double mutants.

Bright-field images of the 3T3-L1 cells overexpressing GFP, Rab5:Q79L, or Rab5:Q79L double mutants revealed parallel results in comparison with the quantitative ORO results (Figure 15 &16).

To further examine the specific molecular requirement for the effect of Rab5 on the differentiation of 3T3-L1 pre-adipocytes, I investigated the effect of deletion of the N-/C-terminal of Rab5 or the Rab5 mutants. These N-/C-terminal residues are essential for Rab5 function. The N-terminal is critical for efficient geranylgeranylation of the C-terminal of Rab5 (Sanford *et al.*, 1995). The C-terminal contains a CCXX motif that is responsible for the post-transcriptional modification, geranylgeranylation, of Rab5 (Farnsworth *et al.*, 1994). The biological activity of Rab5: Δ N19 and Rab5: Δ C4 deletion mutants were examined (Figure 17.A). In comparison with Rab5:S34N, the mutant Rab5:S34N- Δ N19 resulted in a partial, though not significant, loss of differentiation of the 3T3-L1 pre-adipocytes. For the C-terminal deletion, the elimination of the entire CCXX motif (Rab5:S34N- Δ C4) completely abolished the effect of Rab5:S34N on the differentiation of 3T3-L1 pre-adipocytes. In Figure 17, the introduction of the C-terminal deletion in Rab5:Q79L mutant (Rab5:Q79L- Δ C4), but not the introduction of the N-terminal deletion (Rab5:Q79L- Δ N19), reversed the inhibitory effect of Rab5:Q79L mutant on differentiation of 3T3-L1 pre-adipocytes. These data suggest that post-translational isoprenylation modifications of Rab5 are essential for its biological function during differentiation of 3T3-L1 pre-adipocytes.

Figure 17. Selective effect of Rab5 constructs on the differentiation of 3T3-L1 pre-adipocytes.

3T3-L1 pre-adipocytes overexpressing GFP or Rab5 constructs (Rab5:S34N, Rab5:S34N-ΔC4/ΔN19, Rab5:Q79L and Rab5:Q79L-ΔC4/ΔN19) were differentiated as described in Material and Methods. A. Relative lipid content of differentiated 3T3-L1 cells overexpression GFP or Rab5 constructs. 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). B. Representative Western blot of the content of GFP, Rab5 proteins.

II.4.3 Rab5 plays an irreplaceable role in the regulation of adipogenesis

Based on the results collected so far, Rab5 played a regulatory role in adipogenesis. Since the primary role of Rab5 is to promote early endosome fusion in the membrane trafficking process and receptor-mediated endocytosis, I questioned if the other Rab family proteins had their particular roles in the process of adipogenesis. Thus, several small GTPases, including Rab4, Rab7, Rab11, Rab22, and Rab31, as well as the dominant-negative forms (e.g., Rab4:Q67L, Rab7:Q67L, Rab11:Q70L, Rab22:Q64L, Rab31:Q64L) and the dominant-positive forms (e.g., Rab4:S22N, Rab7:S22N, Rab11:S25N, Rab22:S19N, Rab31:S19N) were transfected into the 3T3-L1 pre-adipocytes, respectively. As introduced above, each of these Rab GTPase proteins exhibits particular function in the endocytic pathway, and the active/inactive form of these Rab proteins affect the endocytic pathway in different manners.

In Figure 18, unlike the Rab5:Q79L, none of the Rab GTP-hydrolysis defective mutants (the Rab:QL mutants) tested here inhibited the differentiation of 3T3-L1 pre-adipocytes. Furthermore, none of the Rab GTP-binding defective mutant (the Rab:SN mutants) increased the accumulation of lipids. In addition, the overexpression of Rab:WT proteins did not affect the differentiation process. However, it is also important to note that the overexpression of Rab4:Q67L mutant showed a modest but statistically significant increased accumulation of lipids, while the overexpression of Rab4:S22N decreased it. These data suggest that increasing the levels of Rab5 in the GTP-bound form by overexpressing the Rab5:Q79L mutant and to some extent, overexpressing of Rab5:WT, has a

detrimental and non-substitutable role on the differentiation of 3T3-L1 pre-adipocytes. Furthermore, the data suggest that the Rab5 GDP-bound form plays a particular and significant role in the differentiation process.

Figure 18. A. Selective effect of Rab proteins on the differentiation of 3T3-L1 pre-adipocytes.

3T3-L1 pre-adipocytes overexpression different Rab:WT, Rab:SN, and Rab:QL were differentiated into adipocytes in the presence of induction media, and the accumulation of lipid droplets was measured by the incorporation of ORO 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). B. Representative Western blot of the content of GFP, Rab p, and GAPDH proteins.

II.4.4 Rab5 performs its function via the insulin signaling pathway during the adipogenesis process

Based on the observations above, Rab5, not the other Rab proteins (e.g., Rab7, Rab11, Rab22, and Rab 31), uniquely affects the expression of adipogenesis-associated transcription factors. Then, I wondered what effect of Rab5 constructs overexpression on the expression of other factors (e.g., insulin receptor, GLUT4 and Akt) during adipogenesis would be. This is because the IR, GLUT4, and Akt (the key protein in the PI3K/Akt signaling pathway triggered upon insulin stimulation) have been showed to play a key role during the differentiation of 3T3-L1 pre-adipocytes. Not surprisingly, the expression of the IR, GLUT4, and Akt exhibited an increase in cells overexpressing the Rab5:S34N mutant (Figure 19, Figure 20, and Figure 21). These data further supported the increase of lipids formation observed in this Rab5 mutant cell line (Figure 12). It is important to note that not only the expression level of IR and GLUT4 were increased in Rab5:S34N cell lines, but also the relative phosphorylated IR level was enhanced at day 9 of differentiation (Figure 19, Figure 21). An increase in the phosphorylation of Akt, but not the phosphorylation of Erk1/2 was also observed upon insulin stimulation (Figure 20). Taken together, the data suggest that the overexpression of the Rab5:S34N mutant, which constitutively inhibited the biological function of Rab5 selectively contribute to the up-regulation of expression as well as phosphorylation of the IR by diminishing the internalization of the receptor. This impaired internalization in turn augment the level of phosphorylated Akt and enhance the translocation of GLUT4 to the plasma membrane. Therefore, it is possible to

speculate that Rab5 GTP/GDP ratio has an essential role on differentiation of 3T3-L1 pre-adipocytes.

Figure 19. Effect of Rab5 and the Rab5 mutations on the expression and phosphorylation of the IR.

3T3-L1 pre-adipocytes overexpressing GFP or Rab5 constructs were differentiated as described in Material and Methods. The endogenous levels of IR, phosphorylated-IR (p-IR), and GAPDH proteins were analyzed with specific antibodies by immunoblotting as indicated in the figure upon insulin stimulation. Data represent the mean $\pm$ S.E.M. of three independent experiments. Inset: Representative Western blot of the content of IR, phospho-IR, and GAPDH proteins. * $P < 0.05$ by Student's t-test. A. Relative IR expression level (as compared with GAPDH) of GFP- or Rab5 constructs-overexpressed 3T3-L1 cells. B. Relative p-IR expression level (in comparison with IR) of GFP- or Rab5 constructs-overexpressed 3T3-L1 cells. C. Representative Western blot of the content of IR, phospho-IR, and GAPDH proteins.

Figure 20. Effect of Rab5 constructs on the activation of Akt and Erk1/2.

3T3-L1 pre-adipocytes overexpressing GFP or Rab5 constructs were differentiated as described in Material and Methods. The activation of Akt and Erk1/2 were analyzed with specific antibodies by immunoblotting as indicated in the figure upon insulin stimulation. GAPDH served as loading control. Data represent the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$ by Student's t-test. A. Relative p-Akt level of 3T3-L1 cells expressing GFP or Rab5 constructs. B. Representative Western blot of the content of total Akt, phosphor Akt (p-Akt), and GAPDH. C. Relative p-Erk1/2 level of 3T3-L1 cells expressing GFP or Rab5 constructs. D. Representative Western blot of the content of total Erk1/2, phosphor Erk1/2 (p-Erk1/2), and GAPDH.

Figure 21. Effect of Rab5 and the Rab5 mutants on the expression of GLUT4. 3T3-L1 pre-adipocytes overexpressing GFP or Rab5 constructs were differentiated as described in Material and Methods and the endogenous levels of GLUT4 and GAPDH were analyzed with specific antibodies by immunoblotting as indicated in the figure. A. Relative GLUT4 level in comparison with GFP cells. Data represent the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$ by Student's t-test. B. Representative Western blot of the content of GLUT4 and GAPDH.

II.5 Discussion

In this Chapter, Rab5 has been established to play a detrimental role in the process of adipogenesis with a decreased GTP-bound Rab5 level and without affecting the total expression of Rab5 in the 3T3-L1 pre-adipocytes. Consistent with these observations, I show that increasing the Rab5-GTP level is also detrimental and irreplaceable for the differentiation of 3T3-L1 pre-adipocytes.

First, the expression of different Rab5 isoforms revealed a slight but statistically significant decrease in the accumulation of lipids in the cytosol, which was detected via the ORO staining assay (Figure 10). Interestingly, the deletion of

individual Rab5 isoforms, as well as all three isoforms via RNA interference also resulted in a decrease of lipids accumulation. The Rab5 isoforms exhibit different roles in different cell types. For instance, the overexpression of Rab5B or Rab5A hampered the degradation of EGFR as well as the progression of EGFR from early to late endosomes upon EGF stimulation in Hela cells (Chen *et al.*, 2009). Rab5A is also found to regulate autophagosome formation in the U2OS cells (human bone osteosarcoma epithelial cells). And Rab5C has been shown to enhance β -integrin recycling in EGF-induced cancer cell invasion (Onodera *et al.*, 2012). Since the insulin receptor and the EGFR are both a member of the receptor tyrosine kinases, these data suggest that Rab5 may affect the process of differentiation by impairing the endocytosis process of the IR.

Second, the activated GTP-bound Rab5 has been demonstrated to undergo a graduated decrease over the differentiation of 3T3-L1 cells (Figure 11). The reduction of Rab5 GTP-bound form exhibited a negative relationship with the accumulation of adipogenesis-associated transcription factor, PPAR γ , which served as the marker of adipogenesis process (Figure 11). In addition, the decrease of Rab5 GTP-bound level during adipogenesis happened without any changes in the total endogenous expression level of Rab5 (Figure 11.D). These data suggest that the ratio of Rab5-GTP over total Rab5 may be important for the differentiation of 3T3-L1 pre-adipocytes.

Third, two Rab5 mutants, the dominant-negative Rab5:S34N and the dominant-positive Rab5:Q79L, exhibited opposite roles in the differentiation of 3T3-L1 cells, where the Rab5:Q79L inhibited this process whereas the Rab5:S34N

promoted the process. In addition, the expression of the Rab5:Q79L mutant, but not the Rab5:S34N mutant, blocked this process by inhibiting the expression of PPAR γ and C/EBP α in the 3T3-L1 pre-adipocytes. In previous studies, the expression of Rab5:Q79L mutant results in enlarged endosomes in multiple cell types, whereas the expression of Rab5:S34N inhibited the internalization of the EGFR in Hela cells as well as phagosome formation and *T. cruzi* internalization in the Raw 264.7 mouse macrophage cells (Dinneen & Ceresa, 2004; Galperin & Sorkin, 2003; Li *et al.*, 1994; Maganto-Garcia *et al.*, 2008; Stenmark *et al.*, 1994). In the confocal images, enlarged endosomes can be observed in the Rab5-Q79L-overexpressed cells, which suggests that GTP-Rab5 may diminish the accumulation of lipids by impairing the endocytosis process. Furthermore, Rab5:Q79L- Δ C4, a mutant lacking CCXX (X refers to any amino acid) motif that is required for isoprenylation, completely reversed the inhibitory effect of Rab5. Previously, researchers show that the N-terminal is critical for efficient geranylgeranylation of the C-terminal of Rab5, whereas the C-terminal contains a CCXX motif that is responsible for the post-transcriptional modification of Rab5 (Farnsworth *et al.*, 1994) (Sanford *et al.*, 1995). My data suggest that the C-terminal is essential for the function of Rab5 in regulating the differentiation process. Though the N-terminal supports the function of the C-terminal, deletion of the N-terminal alone does not have a significant effect on the differentiation of 3T3-L1 pre-adipocytes.

Fourth, the overexpression of Rab5:Q79L-K116E, but not the other Rab5:Q79L double mutants (Rab5:Q79L-Y57A, Rab5:Q79L-W74A, Rab5:Q79L-

Y82A, Rab5:Q79L-Y89A, or Rab5:Q79L-R120E), significantly promoted the accumulation of lipids in the differentiated 3T3-L1 cells (Figure 15). Previously, the Y57A, W74A, Y82A, and Y89A were predicted to be decisive binding epitopes for EEA1 and Rabenosyn-5, and the Rab5:Q79L-Y57A, Rab5:Q79L-W74A, Rab5:Q79L-Y82A, and Rab5:Q79L-Y89A mutants all showed reduced binding affinity to Rab5 effector Rabaptin-5 (G Zhu *et al.*, 2004; Mishra *et al.*, 2010). The K116 serves as a mono-ubiquitination site of Rab5 (Wagner *et al.*, 2011). But the Rab5:K116R mutant did not affect the recruitment of Rab5 effectors or alter the GTP loading/GDP release activities of Rab5 (Shin *et al.*, 2017). My data suggested a novel role of the K116 residue in regulating the function of Rab5 in adipogenesis process.

Fifth, several small GTPases (e.g., Rab4, Rab7, Rab11, Rab22, and Rab31) were unable to affect this differentiation process as compared with Rab5. Though each of the tested Rab proteins exhibits particular functions in different steps of endocytosis (Frittoli *et al.*, 2014; Lodhi *et al.*, 2007; Takahashi *et al.*, 2012; Vanlandingham & Ceresa, 2009; HP Zhu *et al.*, 2009). None of the chosen Rab5 proteins displayed a similar role as Rab5 in the differentiation process.

Sixth, both the expression levels of insulin receptor and GLUT4 were up-regulated under the overexpression of Rab5:S34N and down-regulated under the overexpression of Rab5:Q79L. Besides, the levels of phosphorylated Akt and phosphorylated insulin receptor were increased in the Rab:S34N cells.

These observations can be explained, at least in part, by the fact that Rab5:Q79L, but not Rab5:S34N mutant, increased internalization of the insulin

receptor, thus, decreasing both cell surface localization as well as tyrosine autophosphorylation of the insulin receptor at day 9 day of the differentiation process. Consistent with this idea, internalized and less active insulin receptor will not be able to fully activate Akt, a key molecule required for the activation of GLUT4. These molecules have been implicated in the differentiation of 3T3-L1 pre-adipocytes.

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CHAPTER III

III. Role of Rab5 regulators and effectors in the process of adipogenesis

III.1 Introduction

Adipogenesis is a complicated process triggered by hormones and governed by corresponding signaling pathways (Chi *et al.*, 2017; Hinoi *et al.*, 2014; Nam *et al.*, 2015; Wu *et al.*, 2015). The insulin signaling pathway plays an essential role in multiple cellular processes, including cell proliferation, cell migration, and cell differentiation, in a wide range of cell types (Huttala *et al.*, 2016; Shukla *et al.*, 2009; Yen *et al.*, 2015). Specifically, insulin is proved to be the most potent hormone to trigger the differentiation of the pre-adipocytes (Klemm *et al.*, 2001). The dominant effects of insulin on the pre-adipocytes coincide with the high expression of IR on the plasma membrane of the pre-adipocytes, with a number of about $200\sim300 \times 10^3$ per pre-adipocyte compared to ~40 per erythrocyte (Watanabe *et al.*, 1998). The binding of insulin to the IR triggers autophosphorylation of several tyrosine sites on the intracellular portion of the IR β -subunit (Saltiel & Kahn, 2001). The insulin signaling pathway activated upon insulin stimulation governs the body metabolism by affecting the rates of glucose transport, glycogen synthesis, fatty acid synthesis, and enzyme/protein synthesis, and the expression of adipocyte differentiation-associated proteins (Dimitriadis *et al.*, 2011). Under the stimulation of insulin, the IR undergoes a rapid internalization process ($t_{1/2} < 3$ min) through the receptor-mediated endocytosis (RME), in which the insulin-IR complex is engulfed into the cell with a region of the plasma

membrane as a clathrin-coated pit (CCP) in an insulin concentration-dependent manner (Jochen *et al.*, 1989; Marshall, 1985). Internalized CCP is then delivered through the intracellular membrane trafficking system, in which the CCP is transport to the early endosome, the late endosome, and eventually the lysosome to be degraded or the plasma membrane to be reused (Scheidel *et al.*, 2015).

The small GTPase Rab5 is dynamically involved in several cellular trafficking and signaling events, including receptor internalization, fusion of endocytic vesicles with early endosomes, and signaling to the nucleus (Barbieri *et al.*, 2000; Bucci *et al.*, 1992; GP Li *et al.*, 1994). As is described in Chapter II, Rab5 regulate the adipogenic differentiation of the pre-adipocytes through the insulin signaling pathway. Specifically, the overexpression of the GTP-binding defective mutant of Rab5 (Rab5:S34N) increases differentiation of the 3T3-L1 pre-adipocytes, whereas the overexpression of the GTPase-defective mutant of Rab5 (Rab5:Q79L) improves differentiation of 3T3-L1 pre-adipocyte. Besides, previous study shows that the formation of Rab5-positive endosomes and the activation of Rab5 upon insulin in intact cells were both blocked by the treatment of a receptor tyrosine kinase inhibitor (Jozic *et al.*, 2011). In addition, the impaired Rab5 activation can be rescued by the overexpression of Rab5:Q79L, which parallels with the fact that the formation of Rab5:Q79L mutant-positive endosomes is not affected by the receptor tyrosine kinase inhibitor (Jozic *et al.*, 2011).

Rab5 cycles between the active GTP-bound form and the inactive GDP-bound form to serve as the molecular switch in the early endosome fusion process (G Li, 2015). The activation of Rab5 is regulated by a number of guanine nucleotide

exchange factors (GEFs). All these proteins contain a highly conserved VPS9 domain that catalyzes nucleotide exchange on GTP-bound form Rab5. The Rab5 GEFs include Rabex-5, Alsln, the RIN family proteins, RAP6 (which is also known as RME-6 and GAPex-5), and Rin1 (Han & Colicelli, 1995; Horiuchi *et al.*, 1997; Hunker, Galvis, *et al.*, 2006; Kajiho *et al.*, 2003; Otomo *et al.*, 2003; Saito *et al.*, 2002; Woller *et al.*, 2011). Furthermore, it seems that these Rab5 GEFs also interact with several receptors via diverse mechanisms (Barbieri *et al.*, 2003; Deininger *et al.*, 2008; Penengo *et al.*, 2006; Su *et al.*, 2007; Tall *et al.*, 2001). The GTPase activating proteins (GAPs) convert Rab5 to its GDP-bound form. Two well-characterized Rab5 GAPs are RabGAP-5 and RN-tre. The deletion of RabGAP-5 or RN-tre increase the level of active Rab5 *in vitro* (Haas *et al.*, 2005; Palamidessi *et al.*, 2013).

One of the best-characterized Rab5 GEFs is RIN1 (Ras interference 1), a multifunctional protein composed of several functional domains, including an SRC homology 2 (SH2) domain, a proline-rich (PR) domain, a vacuolar protein sorting 9 (VPS9) domain, and a Ras association (RA) domain (Balaji *et al.*, 2014). Previous work suggested that RIN1 is an exchange factor for the small GTPase Rab5, whose overexpression stimulates IR-mediated endocytosis. And it has shown that deletion of the RIN1 N-terminal, which contains the SH2 domain and PR domain of RIN1, blocked the insulin stimulated receptor-mediated endocytosis (Hunker, Giambini, *et al.*, 2006). Specific residues, such as RIN1:D537A and RIN1:Y561F on the RIN1-VPS9 domain are critical for Rab5 activation, whereas RIN1-R94A on the RIN1-SH2 domain is crucial for the interaction between EGFR

and RIN1 upon EGF stimulation (Barbieri *et al.*, 2003; Galvis, Balmaceda, *et al.*, 2009; Galvis, Giambini, *et al.*, 2009). Thus, relationship of key residues required for optimal RIN1 functionality is critical in order to understand the connection between receptor signaling and trafficking.

The Rabex-5 protein structure is identified to be made up of an N-terminal domain, a membrane binding domain, a helical bundle (HB) domain, a VPS9 domain, a coiled-coil (CC) domain, and a PR domain (Shin *et al.*, 2012). Besides the well-studied VPS9 domain to interact and activate Rab5, the HB domain has been found to play a role in the association with early endosomes and activating Rab5 *in vivo* (Zhu *et al.*, 2007). The GEF activity of Rabex-5 is initially autoinhibited by its CC domain, which binds weakly to the substrate-binding site of the VPS9 domain (Zhang *et al.*, 2014). When the Rabex-5 activates Rab5 and recruits Rabaptin-5 (a Rab5 effector), Rabaptin-5 will in turn bind to the GTP-bound Rab5 and recruits Rabex-5 in a positive loop. The binding with Rabaptin-5 displaces the CC domain of Rabex-5 thus exposes a larger substrate-binding site for Rab5, causing the release of the GEF activity (Zhang *et al.*, 2014). The autoinhibition of Rabex-5 explains why in comparison to other well-characterized small GTPases and their corresponded GEFs, Rabex-5 exhibits a relatively lower affinity to Rab5 (Esters *et al.*, 2001). The autoinhibition also gives a clue to why the GTP exchange activity of Rabex-5 is much higher when in the Rabex-5-Rabaptin5 complex (Delprato *et al.*, 2004). Besides, the VPS9 domain of Rabex-5 alone also shows a higher GTP exchange activity than the full-length Rabex-5 (Delprato *et al.*, 2004). Thus, Rabex-5 is involved in early endosome fusion as a Rab5 activator with

regulated activity. Thus, the GTP exchange activity of Rabex-5 is much higher when in the Rabex-5-Rabaptin5 complex (Delprato *et al.*, 2004). A study has shown that the Ras ubiquitination been mediated by Rabex-5 promoted Ras endosomal localization and led to the suppression of Erk activation (Xu *et al.*, 2010). Interestingly, the VPS9 domain of Rabex-5 alone shows a higher GTP exchange activity than the full-length Rabex-5 (Delprato *et al.*, 2004).

In the processes of vesicle formation and delivery, Rab5 is also regulated by multiple Rab5 effectors. As one of the best studied Rab proteins to date, Rab5 has been found to interact with as many as 30 different effector proteins (Pfeffer, 2005). For instance, a novel Rab5 effector, VPS34, is classified as the Class III enzyme of the phosphoinositide 3 kinase (PI3K) family and only uses phosphatidylinositol as substrate to generate phosphoinositide (PI) at the 3-position of the inositol ring, which is named the phosphatidylinositol 3-phosphate (PI3P) (Backer, 2008). Since PI3P is required for the recruitment of EEA1 and Rabenosyn-5 to the active form of Rab5, VPS34 plays a positive role in early endosome fusion and the endocytosis process (Siddhanta *et al.*, 1998) (Christoforidis, Miaczynska, *et al.*, 1999). EEA1, an important Rab5 effector that binds to Rab5-GTP form, is the only effector that is necessarily required by minimal fusion and mediates endosome docking (Christoforidis, McBride, *et al.*, 1999). Though Rabenosyn-5 is also recruited to the early endosome in a PI3P-dependent manner, it does not bind to GTP-bound Rab5 directly but is required for fusion of the plasma membrane-derived CCVs with early endosomes, as well as for homotypical early endosome fusion (Nielsen *et al.*, 2000). Another Rab5 effector,

Rabaptin-5, was initially identified as an GTP-bound Rab5 effector through a yeast 2-hybrid screen (Stenmark *et al.*, 1995). Rabaptin-5 is mostly found as a complex with Rabex-5 in the cell (Horiuchi *et al.*, 1997). Rabex-5 and Rabaptin-5 recruit each other in a positive loop upon Rab5 activation and thus keep the membrane Rab5-positive until the loop is interrupted by other recruited compartments (Kalin *et al.*, 2015). Deletion of the Rab5-binding domain of Rabaptin-5 results in giant endosomes as well as impaired endocytosis process (Kalin *et al.*, 2015).

In this project, I examined the effect of Rab5 GEFs (e.g., Rabex-5 and RIN1), Rab5 GAPs (e.g., RN-tre), and Rab5 effector (e.g., VPS34) on adipogenesis. In addition, I also elucidated the determinant residues and domains of RIN1 during adipogenesis process. Based on the determined specific residues and domains of RIN1 that are required for signal transduction during adipogenesis, and the *in silico* docking analysis between the IR and RIN1, a model of RIN1 function on the insulin signaling pathway during adipogenesis is proposed.

III.2 Specific aims and research design

III.2.1 Specific aim 2: To determine the effect of Rab5 regulators on adipogenesis.

Rab5 effectors denote a set of proteins that interact with the GTP-bonded Rab5. Each effector mediates a feature of Rab5 function and thus affects different aspects of downstream signaling. For example, Rabaptin-5 is involved in membrane fusion, it interacts with GTP-bonded Rab5 and stabilizes Rab5 in its active form. VPS34 generates PI3P, which is required for the recruitment of EEA1

and Rabenosyn-5 to the active form of Rab5. These recruitments allow both EEA1 and Rabenosyn-5 then take part in the molecular machinery of regulating endosome fusion. The function of Rab5 dramatically depends on the recruitment of different effectors to clear several downstream trafficking and signaling events.

One of the best-characterized Rab5 GEF is RIN1, which is required for the activation of Rab5 upon ligand stimulation in several cellular processes. The expression of RIN1:Wild-type, but not its depletion, affects the nucleotide status of Rab5. Preliminary results indicated that RIN1 interacted with the activated insulin receptor. Thus, I elucidated

- 2.a. whether the expression (or depletion) of Rab5 GAPs (RN-tre, RabGAP-5), Rab5 GEFs (RIN1, Rabex-5, and RAP6), or Rab5 effector (VPS34) affected adipogenesis.
- 2.b. the domains of Rab5 regulators required for adipogenesis.
- 2.c. the specific effect of the expression of RIN1 and its mutants on adipogenesis.
- 2.d. the key residues on RIN1 and the insulin receptor in the IR – RIN1 interaction.
- 2.e. a 3-dimensional model of the interaction between the VPS9-HB domain of RIN1 and the IR cytoplasmic tail.

III.2.2 Research design 2

Cell lysates of the 3T3-L1 cells that have been transfected with GFP, RIN1, Rabex-5, RAP6, RabGAP-5, and RN-tre were incubated with GST-labeled EEA1. The bonded proteins were eluted and be used for immunoprecipitation analysis.

Then the cDNA of RIN1:WT and RIN1 mutants were cloned into the pMX-puro vector at EcoRI and NotI restriction sites and plasmid DNA were purified via the plasmid purification assay. The purified DNA was then transfected into the Plat-A cells using Lipofectamine® 2000. After 48 hours, virus suspension from the Plat-A cells were applied to the 3T3-L1 mouse pre-adipocyte cell line for the expression of RIN1 or RIN1 mutants. The transfected cells were then selected with puromycin, and used for several biological assays, including cell cycle testing, lipid accumulation testing (ORO assay, only for differentiated cells) or western blotting.

The RIN1:WT, RIN1:VPS9, RIN1 mutants and RIN1:VPS9 mutants were cloned into the pB42AD vector at the EcoRI and XhoI restriction sites. The insulin receptor tail (IR- β) and the catalytic defective mutant of the insulin receptor were cloned into the pLex-A vector at the EcoRI and BamHI restriction sites. All plasmids were purified and transfected into the yeast EGY48 strain. One pLex-A plasmid was combined with one pB42AD plasmid and co-transfected into the yeast cells. The co-transfected yeast was used for yeast β -Galactosidase Assay to test the intensity of protein-protein interaction. At last, the data were input into the software, i.e., the SWISS MODEL server and the PyMOL, to build the 3-dimensional structural model of the interaction between the RIN1:VPS9 and the IR-tail.

III.3 Materials and Methods

Methods that have been described in the previous chapter are not shown in this section.

III.3.1 Materials

The 3T3-L1 pre-adipocyte cell line was obtained from the Zen-Bio, Inc. (NC, USA). The Platinum A (Plat-A) retroviral packaging cell line and the pMX-puro retroviral vector were purchased from Cell Biolabs, Inc. (San Diego, CA, USA). The yeast *Saccharomyces cerevisiae* strain EGY48 was obtained from American Type Culture Collection (ATCC, Manassas, CA, USA). The pLex-A and pB42AD control vectors were purchased from Addgene (Cambridge, MA, USA). Yeast minimal SD Agar base, yeast minimal SD base, yeast dropout supplements were purchased from Takara Bio, Inc. (Mountain View, CA, USA). Primary antibodies, including the Akt1, p-Akt (Ser473), PPAR γ , C/EBP α , Erk1/2, p-Erk1/2, FABP4, FAS, ACC, GAPDH, and Rab5, and secondary antibodies, including the anti-Rabbit IgG AND anti-Mouse IgG, were purchased from the Cell Signaling Technology (Boston, MA, USA). Fetal bovine serum (FBS) was purchased from the RMBIO, Inc. (Missoula, MT, USA). Other reagents and supplies, if not specifically described, were purchased from Thermo Fisher Scientific (Weston, FL, USA).

III.3.2 cDNA Construction.

Complementary DNAs (cDNA) of green fluorescent protein (GFP), GFP-Rab5:wild-type (GFP-Rab5:WT), and RIN1:WT were cloned into the pMX-puro vector at the EcoRI and NotI restriction sites (Onishi *et al.*, 1998). cDNA of IR β -subunit intracellular tail (IR- β) was cloned into the pLex-A vector at the EcoRI and BamHI restriction sites. cDNA of RIN1:WT and the C-terminal of RIN1 containing both the VPS9 and RA domains (R3:WT) were cloned into the pB42AD vector at the EcoRI and XhoI sites. RIN1-SH2 mutants (RIN1:W69F, RIN1:A76F, RIN1:R94A, RIN1:H120F, RIN1:121F, RIN1:Y148F) conjugated the pB42AD vector were purchased from GenScript, Inc. (Piscataway, NJ, USA). IR-kinase dead (IR:KD, IR:K1030A) and IR- β mutants (IR:Y972F, IR:Y1158F, IR:Y1328F, and IR:Y1334F) on the pLex-A vector, RIN1-SH2 mutants and RIN1-VPS9 mutants (RIN1:Y537F, RIN1:Y561F, RIN1:T580A) on the pMX-puro vector, and R3 mutants (R3:Y506F, R3:Y523F, R3:Y537F, R3:P541A, R3:Y561F, R3:Y572F, R3:Y577F, R3:Y578F, R3:T580A, R3Y:Y629F) on the pB42AD vector were made via the Q5[®] Site-Directed Mutagenesis Kit purchased from New England Biolabs, Inc. (NEB, Ipswich, MA, USA).

III.3.3 siRNA sequences and transfection.

RNA interference (RNAi) directed against:

mouse RIN1 [5'-UUUAUACAUUUGCUUCACACCUAAGC-3'],

mouse Rabex-5 [5'-UUUAUAGAGACGCGUCAUGAUGUGC-3'],

mouse RAP6 [5'-AAGAATCGATTACCTATAGCA-3'],

mouse Rn-tre [5'-TAGACAGTATAATCACGCA-3'],
or mouse RabGAP-5 [5'-GGACGACTCTGTGACAGAAGGAGTA-3'] and [5'-GAACATCAAGCAAACAGAGCTGGTA-3'] were designed and synthesized by Ambion (Austin, TX). A scrambled siRNA sequence was designed as a control [5'-CACCUAUCCGUGGUUCAA-3']. Prior to siRNA sequence transfection, cells were plated in growth medium without antibiotics at 70-80% confluence. siRNA sequence (20 nM final concentration) transfection was performed using Lipofectamine-2000 as specified by Invitrogen (Invitrogen, Carlsbad, CA). After transfection, cells were used for the indicated assay (e.g., for immunoblotting, differentiation or pull-down assays).

III.3.4 Cell counting assay.

The 3T3-L1 pre-adipocytes under different treatment (e.g., with or without treatment of the differentiation media) and at different time points during differentiation (e.g., 0 h time point before the treatment of differentiation media, 15 minutes/24 hours/48 hours time points after the treatment of differentiation media) were detached by trypsin and diluted with DMEM. 100 µl of the cell suspension will be mixed with 100 µl of the Trypan Blue solution. 10µl of the mixture was applied to a hemocytometer or a TC20™ Automated Cell Counter (Bio-Rad, Inc.) to be counted.

III.3.5 Yeast 2 hybrid system.

III.3.5.1 Yeast transformation

Protein-protein interactions were tested and analyzed by the yeast 2-hybrid system (Y2H). The generation of cDNA-containing vectors were described above. These pectors were transformed into the yeast stain EGY48 containing a pSH18-34 reporter vector. A cDNA-containing pB42AD vector and a cDNA-containing pLexA vector were co-transformed into yeast cells by the Yeast Transformation Kit. Transformed yeast cells were selected by SD agar plates that lack leucine (for selection of pSH18-34), histidine (for selection of pLexA), tryptophan (for selection of pB42AD) and uracil (for selection of yeast stain EGY48).

III.3.3.2 Yeast β -galactosidase Assay

The protein-protein interaction was quantified by the Yeast β -galactosidase (β -gal) Assay. Yeast cells were collected in the log phase followed by three freeze-thaw cycles and lysed in 70 μ l of Y-PER buffer (50 mM Tris-HCl, pH 8; 50 mM NaCl; 5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; 5 mM DTT). The β -gal reaction was triggered by adding 70 μ l of 2* β -gal buffer. Then samples were incubated in 37°C until an obvious yellow color showed up. At last, 56 μ l of stop solution was added and samples were tested under 420 nm wavelength. The β -gal activity can be calculated by the formula below:

$$\beta\text{-Gal activity} = \frac{1000 * OD_{420}}{t * V * OD_{660}} \quad (t = \text{total incubation time, } V = 0.196 \text{ ml})$$

III.3.6 *In silico* Studies

The homology model of the VPS9 domain with N-terminal helical bundle of RIN1 was built by the SWISS-MODEL (Arnold et al., 2006) using the crystal structure of the VPS9 domain of Rabex-5 (Pdb Id:1TXU) as a template (Delprato et al., 2004). The model was built from residue M350 to L612 of RIN1. The sequence identity between the VPS9 domain of RIN1 and Rabex-5 is 30.0%. The coordinate of Rab5 (1R2Q) was taken from the protein data bank (Terzyan et al., 2003; Zhang et al., 2014). The RIN1:VPS9-Rab5 complex was built using the crystal structure of the complex of Rab5 and Rabex-5 (Pdb Id: 4Q9U) (Zhang et al., 2014). The RIN1:VPS9-Rab5 complex was minimized by using NAMED and VMD (Humphrey et al., 1996; Phillips et al., 2005). The whole RIN1 model was built by using RaptorX structure prediction server (Kallberg et al., 2012). The server used the SH2 domain of human carboxyl-terminal Src kinase (Pdb Id: 3EAC) (Liu & Cowburn, 2016), Rabex-5 (Pdb Id: 2OT3) (Delprato & Lambright, 2007) and the RA domain (Pdb Id: 4K81) (Qamra & Hubbard, 2013) as templates. The molecular model was drawn with PYMOL (Delano, 2001).

III.3.7 Statistical analysis

All experiments presented were repeated a minimum of three times. Normal distributed data were represented in mean $\pm$ standard deviation (Wilfling et al.). The difference between groups were determined by the Student t-test. Tests were considered statistically significant if p-value were less than 0.05.

III.4 Results

In chapter II, Rab5 has been established to play a detrimental role in the process of adipogenesis with a decreased GTP-bound Rab5 level. However, the total expression of Rab5 in the 3T3-L1 pre-adipocytes was not affected. The overexpression of the Rab5:Q79L mutant, which increased GTP-bound Rab5 level due to its low GTP hydrolysis rate, impaired the accumulation of cytosolic lipid droplets in the 3T3-L1 differentiated cells. Whereas the overexpression of Rab5:S34N, the GTP-binding defective mutant that maintained Rab5 in its inactive form, promoted the formation of lipid droplets in the differentiated 3T3-L1 cells. Besides, a gradient decrease of GTP-bound Rab5 was observed during the 3T3-L1 cell differentiation process in a time-dependent manner. Thus, I questioned if the Rab5 GEFs, Rab5 GAPs, and Rab5 effectors participate in the regulation of Rab5 during the adipogenesis process.

III.4.1 Rab5 GEFs were down-regulated during the differentiation of 3T3-L1 pre-adipocytes

In order to address whether the Rab5 GEFs participate in the differentiation of adipocytes, cell lysates collected from 3T3-L1 pre-adipocytes at before (day 1) and after (day 9) differentiation were prepared and utilized for immunoblotting. The expression of several Rab5 GEFs, such as RAP6 and Rabex-5, and Rab5 GAPs, such as RabGAP-5 and RN-tre, were analyzed. As described early, GAPDH served as the whole cell lysate loading control. PPAR γ , C/EBP α , acetyl-CoA

carboxylase (ACC), fatty acid synthase (Kralisch & Fasshauer), GLUT4, and IR were adipogenic markers indicating the extent of differentiation.

Figure 22. Representative Western blot of the content of Rab5 GEPs, Rab5 GAPs, typical adipogenic markers, and GAPDH.

3T3-L1 pre-adipocytes expressing GFP were lysed before (day1) and after (day9) of differentiation as described in Material and Methods.

In Figure 22, all adipogenic markers, including PPAR γ , C/EBP α , ACC, FAS, GLUT4, and IR exhibited an increased expression level at day 9 after differentiation in comparison with day 1. The two Rab5 GEFs tested, Rabex-5 and RAP6, showed a decreased expression in mature adipocytes (day 9). However, the two Rab5 GAPs, RabGAP-5 and RN-tre, did not exhibit obvious changes between day 1 and day 9 during differentiation. The results suggest an essential

role of Rab5 GEFs in the regulation of Rab5 nucleotide states during the differentiation process.

III.4.2 The Rab5 GEFs downregulated the accumulation of lipids in the differentiated 3T3-L1 cells.

In order to address the specific role of different Rab5 GEFs (e.g., RIN1, Rabex-5, and RAP6) and Rab5 GAPs (RN-tre and RabGAP-5) in the differentiation of 3T3-L1 cells, the individual overexpression of specific Rab5 GEFs/GAPs in 3T3-L1 cells was accomplished via the pMX-puro retroviral vector system that has been described in the Materials and Methods. And to further explain the role of a specific Rab5 GEF/GAP in regulating the Rab5 nucleotide states, I lysed the 3T3-L1 cells overexpressing RIN1, Rabex-5, RAP6, RN-tre, or RabGAP-5 before (day1) and after (day 9) differentiation. The cell lysates were incubated with GST-EEA1 (glutathione-S-transferase-EEA1) followed by GTP-Rab5 pull-down assay, and the pull-downs were subjected to immunoblotting analysis to determine the ratio of GTP-Rab5 (G-Rab5) to total Rab5 (T-Rab5). Besides, siRNA targeting specific Rab5 GFF and Rab5 GAP, as well as scrambled siRNA as control, were designed and induced into the 3T3-L1 cells to selectively silence a particular Rab5 GEF/GAP. At last, 3T3-L1 cells overexpressing GFP or a specific Rab5 GEF/Rab5 GAP, and 3T3-L1 cells induced with scrambled siRNA or Rab5 GEF/GAP siRNA, were stained with ORO followed by quantification of lipid content.

In Figure 23, I show that the overexpression of RabGAP-5 affected the differentiation of 3T3-L1 pre-adipocytes as evidenced by the increase in the

accumulation of cytosolic lipids. This increase in the accumulation of lipid droplets was consistent with the decreased Rab5-GTP level in cells overexpressing RabGAP-5 as compared with the GFP cells (Figure 23). Specifically, I also observed statistically significant inhibition of the Rab5-GTP level in cell lines expressing RabGAP-5 at both day 1 and 9 as compared with GFP-control cells. Similarly, the overexpression of RN-tre increased adipogenesis (Figure 23). More importantly, RabGAP-5 ($137\pm4\%$) is more active than RN-tre ($121\pm4\%$) during the differentiation process (compare Figure 23.A with Figure 24.A) as well as in the levels of GTP-bound Rab5 (compare Figure 23.C with Figure 24.C). Moreover, the Rab5-GTP level was dramatically reduced in cells expressing Rab5 GAPs, and no further reduction of the Rab5-GTP level was observed at day 9 of differentiation (Figure 23.C and Figure 24.C). To further uncover the role of Rab5 GAPs in the differentiation of 3T3-L1 pre-adipocytes, I utilized specific siRNA to induce the deletion of specific Rab5 GAP in the 3T3-L1 pre-adipocytes. Individual removal of Rab5 GAPs produced a significant inhibition on the accumulation of lipids (Figure 23.B and Figure 24.B). These observations suggest that the decrease of the GTP-bound Rab5 induced by the two Rab5 GAPs has a positive effect on the differentiation of 3T3-L1 pre-adipocytes.

Figure 23. Selective effect of RabGAP-5 on the differentiation of 3T3-L1 pre-adipocytes.

Data represent the mean $\pm$ S.E.M. of three independent experiments. A. 3T3-L1 cells expressing GFP and RabGAP-5 were differentiated. The accumulation of lipid droplets quantified via ORO assay and were represented as relative lipid contents. Inset: Representative Western blot of content of RabGAP-5 and GAPDH proteins. B. Non-transfected cells (Ctl), and cells transfected with scramble RNAi (Scr) or RNAi against RabGAP-5 were differentiated as described. Results were represented as relative lipid contents. Inset: Representative Western blot of content of RabGAP-5, RIN1 and GAPDH proteins. C. Cell lysates of 3T3-L1 pre-adipocytes expressing GFP and RabGAP-5 were prepared at specific day (Day 1 and Day 9) of the differentiation, and the endogenous GTP-bound Rab5 was analyzed by immunoblotting as indicated in the figure.

Figure 24. Selective effect of RN-tre on the differentiation of 3T3-L1 pre-adipocytes.

Data represent the mean $\pm$ S.E.M. of three independent experiments. A. 3T3-L1 pre-adipocytes expressing GFP and RN-tre were differentiated. The accumulation of lipid droplets quantified via ORO assay and were represented as relative lipid contents. Inset: Representative Western blot of content of RN-tre and GAPDH proteins. B. Non-transfected cells (Ctl), and cells transfected with scramble RNAi (Scr) or RNAi against RN-tre were differentiated as described. Results were represented as relative lipid contents. C. Cell lysates of 3T3-L1 pre-adipocytes expressing GFP and RN-tre were prepared at specific day (Day 1 and Day 9) of the differentiation, and the endogenous GTP-bound Rab5 was analyzed by immunoblotting as indicated in the figure.

To gain more information about the regulation of Rab5 cycle during adipogenesis, I analyzed the effect of the expression of Rab5 GEFs (RIN1, Rabex-6, and RAP6) on the differentiation of 3T3-L1 pre-adipocytes (Figure 25~27). In Figure 25, the overexpression of RIN1 blocked the accumulation of lipids. Consistent with this observation, the overexpression of RIN1 increased the Rab5-GTP level as compared with GFP-control pre-adipocytes at day 1 and 9 of differentiation (Figure 25.C). Interestingly, a decrease of the level of Rab5-GTP was observed in RIN1-overexpressed cells at day 9 as compared with day 1 of differentiation, but this reduction in the Rab5-GTP level was less significant as compared with the GFP-control cells (e.g., Rab5-GTP level in GFP-control exhibited a reduction of 75% whereas reduced by only 50% in the RIN1-overexpressed cells) (Figure 25.C).

The effect of the overexpression of Rabex-5 and RAP6 on the differentiation of 3T3-L1 pre-adipocytes were also examined (results are shown in Figure 26 and Figure 27). In Figure 26.A and Figure 27.A, similar to the effect of RIN1-overexpression, the individual overexpression of Rabex-5 and RAP6 blocked the accumulation of ORO by $63\pm 7\%$ and $56\pm 4\%$, respectively. Consistent with these results, I observed that the individual overexpression of either Rabex-5 or RAP6 statistically significant increased the Rab5-GTP level as compared with GFP-control pre-adipocytes at day 1 and 9 (Figure 26.C and Figure 27.C). Furthermore, a statistically significant decrease in the level of Rab5-GTP was also observed in the GFP-overexpressing cells when day 9 was compared with day 1 (a ~75% reduction can be observed). A decrease of the level of Rab5-GTP can also be

detected in Rabex-5- and RAP6-overexpressing cell lines at day 9 as compared with day 1 of the differentiation process. But this reduction between day 9 and day 1 in the Rab5-GTP level was less significant as compared with GFP-control cells (e.g., Rabex-5-cells showed a ~70% decrease whereas the RAP6-cells showed a ~50% decrease) (Figure 26.C and Figure 27.C).

Then the depletion effect of these Rab5 GEFs was examined by inducing specific siRNA on the 3T3-L1 pre-adipocytes during the differentiation process. The down-regulation of each one of the Rab5 GEFs was accompanied by an increase of the accumulation of ORO as compared with control siRNA-induced cells. Specifically, cells overexpressing RIN1 promoted the lipid formation by 26%, Rabex-5 increased lipids accumulation by 20, while the overexpression of RAP6 increased lipids accumulation by 25 (Figure 26.A and Figure 27.A). 3T3-L1 pre-adipocytes differentiation process and also increased the levels of Rab5-GTP as compared with GFP-control cells (data not shown). Taken together, these data suggest that the overexpression or deletion of the specific Rab5 GFE (or the Rab5 GAPs) have essential effects on differentiation of 3T3-L1 pre-adipocytes. The affected differentiation process by these Rab5 GEFs/GAPs were because, at least partially, of their role in regulating the Rab5 nucleotide states.

Figure 25. Selective effect of RIN1 on the differentiation of 3T3-L1 pre-adipocytes.

Data represent the mean $\pm$ S.E.M. of three independent experiments. A. 3T3-L1 pre-adipocytes expressing GFP and RIN1 were differentiated. The accumulation of lipid droplets quantified via ORO assay and were represented as relative lipid contents. Inset: Representative Western blot of content of RIN1 and GAPDH proteins. B. Non-transfected cells (Ctl), and cells transfected with scramble RNAi (Scr) or RNAi against RabGAP-5 were differentiated as described. Results were represented as relative lipid contents. Inset: Representative Western blot of content of RIN1, RIN1 and GAPDH proteins. C. Cell lysates of 3T3-L1 pre-adipocytes expressing GFP and RIN1 were prepared at specific day (Day 1 and Day 9) of the differentiation, and the endogenous GTP-bound Rab5 was analyzed by immunoblotting as indicated in the figure. Inset: Representative immunoblots of samples probed with anti-Rab5 antibodies for total Rab5 (T-Rab5) and active Rab5 (G-Rab5) respectively.

Figure 26. Selective effect of Rabex-5 on the differentiation of 3T3-L1 pre-adipocytes.

Data represent the mean $\pm$ S.E.M. of three independent experiments. A. 3T3-L1 pre-adipocytes expressing GFP and Rabex-5 were differentiated. The accumulation of lipid droplets quantified via ORO assay and were represented as relative lipid contents. Inset: Representative Western blot of content of Rabex-5 and GAPDH proteins. B. Non-transfected cells (Ctl), and cells transfected with scramble RNAi (Scr) or RNAi against Rabex-5 were differentiated as described. Results were represented as relative lipid contents. C. Cell lysates of 3T3-L1 pre-adipocytes expressing GFP and Rabex-5 were prepared at specific day (Day 1 and Day 9) of the differentiation, and the endogenous GTP-bound Rab5 was analyzed by immunoblotting as indicated in the figure.

Figure 27. Selective effect of RAP6 on the differentiation of 3T3-L1 pre-adipocytes.

Data represent the mean $\pm$ S.E.M. of three independent experiments. A. 3T3-L1 pre-adipocytes expressing GFP and RAP6 were differentiated. The accumulation of lipid droplets quantified via ORO assay and were represented as relative lipid contents. Inset: Representative Western blot of content of RAP6 and GAPDH proteins. B. Non-transfected cells (Ctl), and cells transfected with scramble RNAi (Scr) or RNAi against RAP6 were differentiated as described. Results were represented as relative lipid contents. Inset: Representative Western blot of content of RabGAP-5, RIN1 and GAPDH proteins. C. Cell lysates of 3T3-L1 pre-adipocytes expressing GFP and RAP6 were prepared at specific day (Day 1 and Day 9) of the differentiation, and the endogenous GTP-bound Rab5 was analyzed by immunoblotting as indicated in the figure. Inset: Representative immunoblots of samples probed with anti-Rab5 antibodies for total Rab5 (T-Rab5) and active Rab5 (G-Rab5) respectively.

III.4.3 Selective effect of RIN1 and its mutants on insulin signaling during pre-adipocyte differentiation

III.4.3.1 RIN1 overexpression inhibited adipogenesis *in vitro*

Previous results showed that the adipogenic differentiation of Rab5-overexpressed 3T3-L1 cells was promoted. Also, GTP-bound Rab5 were progressively down-regulated over the process of differentiation. In addition, in the previous section, I showed a regulatory role of Rab GEFs in the adipogenesis process. Here, to further examine the effect of RIN1 on the differentiation of 3T3-L1 pre-adipocytes, I overexpressed RIN1:wild-type (RIN1:WT) in the 3T3-L1 cells and tested the effect of RIN1 overexpression on the adipogenic markers (results shown in Figure 28 and Figure 29).

Figure 28. RIN1 effect on the differentiation of 3T3-L1 pre-adipocytes.

A. Relative lipid content of GFP (C+/C-, represent positive control and negative control, respectively) or RIN1:WT transfected 3T3-L1 cells. Cells that were not treated with hormone cocktail served as negative control. Data represent the mean $\pm$ S.E.M. of three independent experiments. *P<0.001 by Student's t-test compared to differentiated GFP-overexpressed cells. S.E.M. = Standard error of the mean. B. Representative Western blot results of RIN1:WT and GFP overexpressed cells.

I used the Plat-A cells to transfer the RIN1:WT or the GFP-containing retrovirus into 3T3-L1 pre-adipocytes. The transfected cells were treated with or without hormone cocktail as was described in Materials and Methods. Then the ORO staining and spectroscopy was used to quantify lipid accumulation in the cytosol (Figure 28). Then the expression of adipogenic transcription factors, PPAR γ and C/EBP α , and adipogenic markers, ACC, FABP4 and FAS in differentiated cells were tested to confirm the ORO results (Figure 29).

Figure 29. Effect of RIN1:WT on transcription factors.

3T3-L3 cells overexpressing GFP or RIN1:WT were differentiated with (positive control) or without hormone inducers.

In Figure 29, the expression level of PPAR γ , C/EBP α , ACC, and FAS in GFP- (cells were treated with or without hormone inducers, which represent the positive control and the negative control, respectively) and RIN1:WT- (cells were hormone treated) overexpressed 3T3-L1 cells were tested via Western blotting. The differentiated GFP-overexpressed cells showed a significantly higher

expression of the two adipogenic transcription factors, PPAR γ and C/EBP α , and the two adipogenic markers, ACC and FAS. However, the differentiated RIN1:WT-overexpressed cells showed a decreased expression of all tested proteins (Figure 29).

These results, showing that RIN1:WT played an inhibitory role in pre-adipocyte differentiation, lead me to further examine the mechanism by which RIN1 affects the adipogenesis process.

III.4.3.2 Two RIN1 selective mutants rescued the inhibitory effects of RIN1 on adipogenesis

RIN1 performs multiple functions in cell metabolism due to its availability of interacting with numerous proteins through different domains. Through its Src homology 2 (SH2) domain, RIN1 interacts with several receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR) and insulin receptor (IR) (Barbieri et al., 2003; Hunker et al., 2006b). Through its VPS9 domain, RIN1 can activate Rab5, a small GTPase that is essential in early endosome fusion process (Tall et al., 2001). Thus, I used two selective mutants, the RIN1:R94A and the RIN1:Y561, to examine the inhibitory role of RIN1 in adipogenesis process. The R94A mutation locates in the SH2 domain of RIN1 and leads a diminished interaction between RIN1 and the IR (Barbieri et al., 2003). The Y561F mutant, however, interferes with the activation of Rab5, resides in the VPS9 domain (Mustafi and Barbieri, 2016).

Again, the ORO staining and spectroscopy assay and the western blot assay were used to quantify the lipid content and to qualify the maturity of differentiated adipocytes, respectively. Interestingly, the overexpression of either mutant could rescue the prohibited effects on adipogenesis caused by RIN1:WT overexpression. In Figure 30, the ORO results presented that the RIN-R94A and RIN1:Y561F mutant increased lipid content from 53% to 77% and 64%, respectively in comparison to the RIN1:WT. The western blot analysis convinced the ORO results where the cells overexpressing either RIN1:R94A or RIN1:Y561F showed a higher expression of the adipogenic transcription factors, PPAR γ and C/EBP α , and two adipogenic markers, ACC and FAS (Figure 30).

Due to the fact that the function-declined RIN1 mutants, either in the SH2 domain or the VPS9 domain, could extricate adipogenesis of 3T3-L1 cells from obstructed effects of RIN1:WT, I suspected that both the SH2 domain and the VPS9 domain are necessary for RIN1 to complete its molecular function in adipogenesis process. As described above, the two mutants are from different domains of RIN1. Thus, the data here suggested that RIN1 affected the adipogenesis process through at least two cellular processes, the endosome pathway and the insulin signaling pathway.

Figure 30. Effect of RIN1 selective mutants on adipogenesis.

A. Relative lipid content of GFP or RIN1:WT transfected 3T3-L1 cells. 3T3-L1 cells overexpressing GFP, RIN1:WT, and RIN1 mutants were treated with or without hormone cocktail as described in Materials and Methods. Cells that were not treated with hormone cocktail served as negative control (C-). (Data represent the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.01$ by Student's t-test compared to differentiated RIN1:WT-overexpressed cells). S.E.M. = Standard error of the mean. B. C. Western blot result of RIN1:WT, RIN1:R94A, RIN1:Y561F and GFP overexpressed cells.

III.4.3.3 RIN1 mutants recovered both Erk1/2 and Akt signaling in compare with RIN1-WT

In the previous study, RIN1 showed co-localization with the IR on both cell membrane and endosomes upon insulin stimulation, and the expression of RIN1-WT blocks the activation of both Erk1/2 and Akt signaling pathway in cancer cells *in vitro* (Hunker, Giambini, *et al.*, 2006). Evidence also showed that both Akt and Erk1/2 signaling reached their maximal level at 15 minutes upon insulin stimulation *in vitro* (Zecchin *et al.*, 2007). Because of the glaring effects of RIN1 and its mutants on the process of adipogenesis and the regulatory role of RIN1 in insulin signaling pathway, I examined the activation of Erk1/2 and Akt, two important proteins in the MAPK and PI3K signaling pathways, respectively, at 15 minutes after hormone treatment (Figure 31). The western blot analysis revealed that both the MAPK and the PI3K signaling pathways were activated upon a 15-minutes hormone treatment in all cell lines (Figure 31). Both activated Akt and Erk1/2 appeared to be downregulated in the RIN1-WT overexpressed cells upon hormone treatment (Fig. 5A) However, the RIN1-R94A overexpressed cells attended to reach a significantly higher signal than the RIN1-WT overexpressed cells at 15 minutes after and even before hormone treatment (Figure 31). Simultaneously, the RIN1-Y561F overexpressed cells presented higher signal of activated Akt (p-Akt) both before and 15 minutes after hormone treatment (Figure 31). The RIN-Y561F mutant increased the Erk1/2 signal before hormone treatment and slightly higher signal after hormone treatment (Figure 31).

Figure 31. The activity of Akt and Erk1/2 before (0) and 15 minutes after hormone treatment.

3T3-L1 cells overexpressing GFP, RIN1:WT, and RIN1 mutants were treated with or without hormone cocktail as described in Materials and Methods. Cells that were not treated with hormone cocktail served as negative control (C-). (Data represent the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.01$ by Student's t-test compared to differentiated RIN1:WT-overexpressed cells). S.E.M. = Standard error of the mean. Representative Western blot of the content of p-Akt and p-Erk1/2 in comparison with GAPDH were shown.

III.4.3.4 RIN1 mutants promote cell proliferation in clonal expansion phase during adipogenesis

Proliferative 3T3-L1 pre-adipocytes were growth-arrested upon contact inhibition before hormone treatment. Prior to terminal differentiation, the hormone triggered growth-arrested cells re-entered cell cycle to perform 2 rounds of post-confluence mitosis, which is named as the clonal expansion phase. Establishing that the RIN1:WT and RIN1 mutants had noticeable effects on the MAPK and PI3K signaling pathways upon hormone treatment in the previous unit, I hypothesized that the ultimate consequences of the protein overexpression would cause notable effects on the clonal expansion phase. proliferation in the early stage of adipogenesis.

Figure 32. Cell proliferation of GFP-, RIN1-, RIN1:R94A-, or RIN1:Y561F-overexpressed cells in the first 48h after hormone induction.

The results indicated a depressed cell growth rate of RIN1 expressed cells while a rescue of cell proliferation when expressing the two selective mutants.

Figure 33. Bright-field images of GFP-, RIN1-, RIN1:R94A-, or RIN1:Y561F-overexpressed cells in the first 48h after hormone induction.

Images were taken under bright-field microscope at 0h (before differentiation) and 48h (after differentiation) with the same magnification of 20X objective lens. The results indicated a depressed cell growth rate of RIN1 expressed cells while a rescue of cell proliferation when expressing the two selective mutants.

To address the effect of RIN1 and its mutants on adipogenesis, I used cell counting assay, both manually and automatically, to intuitively investigate the growth of transfected cells in the first 48 hours upon hormone treatment. In Figure

32 and 33, RIN1 inhibited cell proliferation whereas the two mutants of RIN1 rescued it during the clonal expansion phase of adipogenesis. The results suggest that RIN1 plays an inhibitory role in adipogenesis by, at least partially, impairing the cell mitotic clonal expansion phase of adipogenesis.

III.4.4 The expression of Rab5 effector, VPS34, impaired the differentiation of 3T3-L1 preadipocytes.

Rab5 is regulated by Rab5 GAPs and GEFs, and eventually perform its function by recruiting different Rab5 effectors. In order to investigate the effect of VPS34, a Class III PI3K to generate PI3P upon recruitment by Rab5, I transfected the 3T3-L1 pre-adipocytes with GFP-VPS34 via the pMX-puro retroviral vector system. The transfected cells were treated with or without hormone cocktail as was described in Materials and Methods. Then the ORO staining and spectroscopy was used to quantify lipid accumulation in the cytosol (Figure 34). Then I tested the effect of VPS34 on the insulin signaling pathway by analyzing the phosphorylation levels of Akt and Erk1/2 in the cell lysates. The VPS34- and GFP-transfected cell lysates were collected before differentiation (0h) and 15 minutes after hormone treatment (Figure 35). At last, live cell images were taken under the inverted microscope at the same field of view to analyze the proliferation rate of transfected cells (Figure 36 & 37). Bright-field images were taken at either before differentiation(0h), 15 minutes, 24 hours, and 48 hours (48 hour time point is considered as the end of the mitotic clonal expansion phase) after hormone treatment.

Figure 34. Differentiation of GFP and VPS34 overexpressed cells.

A. Relative lipid content of cells expressing GFP or GFP-VPS34. Data represent the mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$ by Student's t-test. B. Bright-field images of 3T3-L1 cells overexpressing GFP or GFP-VPS34. C. Confocal images of 3T3-L1 cells overexpressing GFP or GFP-VPS34 before differentiation.

In Figure 34.A and B, the ORO quantification assay revealed a significant decreased lipid content of VPS34 overexpressed cells as compared with the GFP positive control (GFP+, cells were treated with hormone inducers during the 10-day differentiation). Interestingly, the VPS34-transfected cells exhibited similar lipid content as the GFP negative control cells which were not treated with hormone inducers during the differentiation process. To my surprise, the size of the VPS34-transfected cells was much bigger than the GFP-transfected cells, which can be observed through both bright-field images and confocal images taken under the same magnification (Figure 34.B & C).

Figure 35. The activity of Akt and Erk1/2 at 0h, 15 min 24h, and 48h after hormone treatment.

3T3-L1 cells overexpressing GFP or GFP-VPS34 were treated with or without hormone cocktail as described in Materials and Methods. Cells that were not treated with hormone cocktail served as negative control. Representative Western blot of the content of p-Akt and p-Erk1/2 in comparison with GAPDH were shown.

In Figure 35, phosphorylated Akt and Erk1/2 were tested at 0h, 15min, 25h, and 48h time points. Surprisingly, the VPS34-transfected cells had an increased activity of both Akt and Erk1/2 as compared with the GFP cells at the same time points. What is more, the VPS34 transfected cells even exhibited a higher phosphorylation base line (compare the activity of both Akt and Erk1/2) and experienced a higher increase at 15min after hormone induction.

Figure 36. The activity of Akt and Erk1/2 at 0h, 15 min 24h, and 48h after hormone treatment.

3T3-L1 cells overexpressing GFP or GFP-VPS34 were treated with or without hormone cocktail as described in Materials and Methods. Cells that were not treated with hormone cocktail served as negative control. Representative Western blot of the content of p-Akt and p-Erk1/2 in comparison with GAPDH were shown.

Figure 37. Bright-field images of GFP and GFP-VPS34 overexpressed cells in the first 48h after hormone induction.

Images were taken under bright-field microscope at 0h (before differentiation) and 48h (after differentiation) with the same magnification of 20X objective lens. The results indicated a depressed cell growth rate of VPS34 expressed cells while a rescue of cell proliferation when expressing the two selective mutants.

As described above, the Akt signaling pathway governs cell metabolism and plays a positive role in 3T3-L1 pre-adipocyte differentiation whereas the Erk1/2 signaling pathway controls gene expression, protein translation, and plays a negative role in promoting the adipogenesis process. Thus, it will be interesting to investigate how the clonal expansion phase is affected during the differentiation

process. In Figure 36 and 37, cell counting data revealed that the expression of VPS34 in the 3T3-L1 cells not only enlarge the size of the pre-adipocytes, but also decreased the cell proliferation rate in the first 48 hours of differentiation.

III.4.5 Selective association of IR with Rab5 GEFs

Based on my observations where the diminishing of the Rab5-GTP levels during the differentiation process is linked to insulin stimulation together with the differential effect of Rab5 GEFs, Rab5 GAPs, and Rab5 constructs on differentiation of 3T3-L1 pre-adipocytes, I concluded that decreasing the Rab5-GTP levels could be related to the inability of Rab5 GEFs and Rab5 GAPs to regulate and/or access to Rab5 proteins during differentiation of 3T3-L1 pre-adipocytes.

To determine the possible cellular mechanism under the regulation of Rab5, I examined whether the IR was associated with specific Rab5 GEFs and/or Rab5 GAPs. 3T3-L1 pre-adipocytes individually expressing each Rab5 GEF and Rab5 GAP were differentiated, and then cells at day 9 of the differentiation process were incubated in the absence or in the presence of insulin for 5 min at 37°C. After the incubation, cells were lysed, and IR was immunoprecipitated followed with immunoblotting analysis with specific anti-Rab5 GEFs antibodies. In Figure 38, I show that RIN1 (Figure 38.A), Rabex5 (Figure 38.B) and RAP6 (Figure 38.C) are able to interact with the IR in the presence of insulin. Results were tested via immunoprecipitation with anti-IR antibodies. In contrast, it was not possible to immunoprecipitate these Rab5 GEFs with IR in the absence of insulin (Figure 38.A,

B, and C). The SH2 domain of RIN1 has been established previously to interact with activated insulin receptor tyrosine kinase domain, which is also confirmed in Figure 38.D. The SH2 domain of RIN1 alone was able to interact with the activated insulin receptor, whereas this domain was not able to interact with the catalytic inactive mutant of the insulin receptor (IR:KD). Interestingly, besides the well-established SH2 domain, another domain of RIN1, the VPS9 domain showed strong interaction with the IR-tail in the yeast two-hybrid system (Figure 38.D). However, the domains that showed higher affinity to the activated IR did not show any binding affinity to the catalytic inactive IR-tail. Taken together, these observations suggest that, IR interacts with Rab5 GEFs only upon the stimulation of insulin. These observations were not surprising for RIN1 since it contains an SH2 domain. However, Rabex5 and RAP6 do not have an SH2 domain, even the association of the latter molecules can be attributed to the requirement of additional factors as it has been shown in the case of the interaction with EGFR. Thus, is reasonable to consider that the VPS9 domain, which is a common domain between these Rab5 GEFs, may be responsible for this interaction with the IR upon insulin stimulation.

Figure 38. Interaction of the IR-cytoplasmic tail and VPS9 containing proteins. 3T3-L1 preadipocytes expressing GFP, RIN1 (A), Rabex-5 (B), and RAP6 (C) were differentiated into adipocytes in the absence (Control) or in the presence of insulin at 37°C for 5 min. After the incubation, cells were lysed and analyzed by immunoprecipitation followed by immunoblotting as described in the figure. Results were represented as relative lipid contents. Data represent the mean $\pm$ S.E.M. of three independent experiments. A./B./C. Representative Western blot of the content of Rabex-5, RAP6, RIN1 constructs, IR, and phospho-IR proteins. * $P < 0.05$ by Student's t-test. D. Yeast cells were co-transformed with plasmids pB42AD fused with Rab5 GEF constructs and plasmids pLexA fused with IR-tail:WT/KD. Co-transformed cells were selected as described in Material and Methods. Liquid cultures in selective media were also assayed for β -Galactosidase (β -Gal) to verify and quantify two-hybrid interactions. The data are presented as means $\pm$ SD of five independent experiments, $n = 5$ * $P < 0.005$. Each independent experiment was performed in quintuplicate.

To further investigate the specificity of the interaction between Rab5 GEFs and IR, I then asked whether a specific domain of Rab5 GEFs was involved in the interaction with the cytoplasmic tail of IR. To address this, I utilized a yeast two-hybrid system to analyze the interaction of the cytoplasmic tail of IR:WT with the following Rab5 GEFs constructs: a) RIN1:SH2, RIN1:VPS9 and RIN1:RA; b) Rabex-5:Z-M (Z: zinc-finger, which displays Ub protein ligase (E3) activity and binds Ub; M: MIU, a motif interacts with ubiquitin), Rabex-5:VPS9, Rabex-5:C-P (Lee *et al.*, 2006); c) RAP6:VPS9, RAP6:PH and RAP6:RGD (Figure 38). The cytoplasmic tail of IR kinase-dead (IR:K1077M), as well as blank vectors that were not conjugated with Rab5 GEF constructs, were used as negative controls. Consistent with previous observations, I found that RIN1:SH2 domain interacted with the cytoplasmic tail of IR (Figure 38.A). However, I also noticed that RIN1:VPS9 domain, but not RIN1:RA domain, interacted with the cytoplasmic tail of IR. Interestingly, the VPS9 domain of RAP6 and Rabex-5, but not other domains from these Rab5 GEFs, also interacted with the cytoplasmic tail of IR (Figure 38.D). These observations, indicates Rab5 GEFs interact with the cytoplasmic tail of IR via VPS9 domain. For additional examination of this novel interaction between the RIN1:VPS9 domain and with the cytoplasmic tail of IR, I built the *in silico* model of the complex of RIN1:VPS9-HB and the cytoplasmic tail of IR using the X-ray crystal structure of the IR and VPS9-HB of Rabex-5. In Figure 34, I show the model of the interaction of VPS9-HB domain and IR-cytoplasmic tail. The molecular analysis of the RIN1:VPS9-HB:IR cytoplasmic tail complex shows that there are 24 residues in RIN1 VPS9-HB and 28 residues in IR-cytoplasmic tail are placed in the interface

between them (Table 4). This complex is being stabilized by hydrogen bonds, van der Waals, hydrophobic and ionic interactions at the interface, and it also predicts that a number of exposed residues in the RIN1; VPS9-HB (Asp537, Pro541, Tyr561, and Thr580) domain. Thr580 places in the α V6 helix on the VPS9 domain and is conserved in hRabex-5, hRIN1, hAlsin, hVarp, but not in hRAP6 proteins. It is placed in the interface, where the side chain of the Thr580 is almost buried into the interface. The side chain of Thr580 in VPS9 domain makes the hydrogen bond interaction with the backbone of Ser1096 of IR cytoplasmic tail. The distance between OG1 atom of Thr580 and OG atom of Ser1096 of IR cytoplasmic tail is nearly 4.5 Å, and it could also form the hydrogen bond between them (Figure 39).

Table 4. In silico analysis of the interaction between the VPS9-HB¹ domain of RIN1 and the IR-tail²

1 The numbering of amino acids of corresponds to the protein of Han and Colicelli (Han & Colicelli, 1995).

2 The numbering of amino acids of IR-B differs from that of the receptor of Ebina et al. by being 47 amino acids greater (Ebina et al., 1985).

Table 4.A Hydrophobic interactions between the VPS9-HB domain of RIN1 and the IR-tail

VPS9-HB Domain	IR-tail
Leu571	Trp1036
Pro599	Leu1085
Leu540	Ile1089, Leu1092
Pro541	Ile1089
Leu588	Ile1089, Leu1092
Ala584	Leu1092
Trp577	Val1097, Phe1175

Table 4.B Cation-Pi interactions between the VPS9-HB domain of RIN1 and the IR-tail within 6 Å

VPS9-HB Domain	IR-tail
Lys520	Tyr1205

Table 4.C Aromatic-aromatic Interactions between VPS9-HB and IR-tail within 4.5 and 7 Å

VPS9-HB Domain	IR-tail
Tyr577	Phe1175

Table 4.D Ionic interactions between VPS9-HB and IR-tail within 6 Å

VPS9-HB Domain	IR-tail
Glu564	Lys1067
Asp537	Arg1086, Glu1090
Lys520	Asp1203, Glu1206
Glu532	Arg1211, Lys1212
Glu530	Lys1212
Glu538	Lys1212, Lys1215
Lys426	Glu1327
Glu574	Glu1328

Table 4.E Hydrogen bond interactions between VPS9-HB and IR-tail.

VPS9-HB Domain	IR-tail	VPS9-HB Domain	IR-tail
Leu571	Phe1101	Asp537	Arg1086, Arg1202
Ala536	Asn1093	Glu530	Lys1212, Lys 1215
Thr572	Lys1173	Glu538	Lys1212, Lys 1215
Glu532	Arg1211, Lys1212, Lys1209	Lys426	Glu1327
Gly533	Lys1212	Lys520	Asp1203, Glu1206
Thr580	Ser1096	Ser563	Glu1035
Arg403	Lys1067, Thr1207	Tyr577	Asp1203
Tyr523	Asp1203

Figure 39. Analysis of the interaction between the IR-cytoplasmic tail and the RIN1:VPS9 mutants.

In silico docking analysis of the IR-cytoplasmic tail and the VPS9-HB domain of RIN1. RIN1:VPS9 domain's residues studied above are shown in magenta sticks and their interactions are shown in separate panels (Inserts). The polar distance less than 4.0 Å are shown in dotted lines. Residues Pro541 and Thr580 of the VPS9 domain are buried in the interface of the complex. The Pro541 makes hydrophobic interaction with Ile1089 of IR-cytoplasmic tail. The side chain of Thr580 makes interaction with the main chain of Ser1096. These mutations of Pro541 and Thr580 to alanine destabilize this complex. The activated IR-cytoplasmic tail structure is taken from the protein data bank (Pdb Id: 4XLV) for docking (Cabail *et al.*, 2015). The homology model of VPS9-HB domain of RIN1 was built by SWISS-MODEL (Arnold *et al.*, 2006) using the crystal structure of Rabex-5 (Pdb id:1TXU) as a template (Delprato *et al.*, 2004). The docking of the IR-cytoplasmic tail and the VPA9-HB domain was done by the ZDOCK online server. The docked model was minimized by using NAMED (Phillips *et al.*, 2005) and VMD (Humphrey *et al.*, 1996). The molecular model was drawn with PYMOL (Delano, 2001).

Tyr561 is also conserved in hRabex-5, hRIN1, hGAPVD1, hVarp but not in hAlsin proteins. It is placed in the α V5 helix, and the side chain is fully buried into the interface between VPS9 and HB domain of RIN1. The hydroxyl group of Tyr561 makes hydrogen bonds with the backbone atom of Tyr506 and Arg455, placed in α H4 (Figure 34). Tyr506 is only present in hRIN1 and it can make a hydrogen bond with the backbone atom of Tyr561. The mutation of Tyr561 and Tyr506 to phenylalanine may lose the hydrogen bond. Based on these observations, I utilized a two-hybrid system to analyze the interaction of the cytoplasmic tail of IR-WT with the following Rab5:VPS9-HB mutants: (Y506F, Y523F, Y537F, P5431A, Y561F, T572A, Y577F, Y578F, T580A) (Figure 39). I found that residues (Tyr506, P541, Y561, and T580) play an important role in the interaction of RIN1:VPS9-HB with the cytoplasmic tail of IR. As expected, the cytoplasmic tail of IR kinase-dead was used as negative control. These observations indicate selective residues of the VPS9-HB domain are required for the interaction of the cytoplasmic tail of IR, which may represent a distinctive and original protein-protein interaction. Taken together, these findings indicate that (a) Rab5 GEFs interact with the cytoplasmic tail of IR, but not with its catalytic inactive mutant, (b) the Rab5 GEFs VPS9 domain is sufficient for this interaction, and (c) Rab5 GAPs do not interact with the cytoplasmic tail of IR. Furthermore, these results also strengthen the possibility that interaction between VPS9 domain of Rab5 GEFs and the cytoplasmic tail of IR, defining a novel and selective interaction between VPS9 domain and the IR cytoplasmic tail.

Figure 40. Analysis of interaction between the IR-cytoplasmic tail (IR-β) and RIN1:VPS9 mutants or IR-β mutants and the RIN1:VPS9 domain.

Yeast cells were co-transformed with plasmids pLexA fused to the IR-β:WT/KD or IR-β mutants (Y972F, IR:Y1158F, IR:Y1328F, and IR:Y1334F) and pB42AD fused to the RIN1:VPS9 or RIN1:VPS9 mutant (Y506F, Y523F, D537A, P541A, Y561F, Y572F, Y577F, Y578F, T580A). Co-transformed cells were spotted onto plates containing selected media and incubated at 30°C as described in Material and Methods. Liquid cultures in selective media were also assayed for β-Galactosidase (β-Gal) to verify and quantify two-hybrid interactions. The data are presented as means ± SD of five independent experiments, n=5 *P<0.05. Each independent experiment was performed in quintuplicate.

Figure 41. Analysis of interaction between the IR-cytoplasmic tail (IR- β) and RIN1:SH2 domain.

Yeast cells were co-transformed with plasmids pLexA fused to the IR- β :WT/KD and pB42AD fused to the RIN1:SH2 or RIN1:SH2 mutants (RIN1:W69F, RIN1:A76F, RIN1:R94A, RIN1:H120F, RIN1:121F, RIN1:Y148F). Co-transformed cells were spotted onto plates containing selected media and incubated at 30°C as described in Material and Methods. Liquid cultures in selective media were also assayed for β -Galactosidase (β -Gal) to verify and quantify two-hybrid interactions. The data are presented as means $\pm$ SD of five independent experiments, $n=5$ * $P<0.05$. Each independent experiment was performed in quintuplicate.

In order to further investigate the interaction between the IR and RIN1, I insert RIN1-SH2:WT, RIN1:VPS9:WT, several RIN1-SH2/VPS9 mutants into the pB42AD vector, and insert the IR- β and IR- β mutants into the pLex-A vector. Figure 40 showed the interaction between IR-cytoplasmic tail (IR- β) and RIN1:VPS9 mutants or IR- β mutants and RIN1:VPS9 domain whereas Figure 41 showed the interaction between the IR- β and RIN1:SH2.

III.5 Discussion

In this section, I show the cellular mechanism of the Rab GEFs and the Rab GAPs in regulating Rab5 function during the differentiation of 3T3-L1 pre-adipocytes. My data indicate that Rab5 inactivation upon insulin stimulation is directly linked to the interaction between the activated insulin receptor and the VPS9 domain of the Rab5 GEFs. The interaction probably affects the availability of Rab5 GEFs to activate Rab5. In addition, the VPS9-IR-tail interaction also affected the recruitment of IRS-1 to the activated insulin receptor cytoplasmic tail.

The expression of Rab5 GEFs, which increased the Rab5-GTP levels, reduced the differentiation of 3T3-L1 pre-adipocytes, while the expression of Rab5 GAPs, which decreased Rab5-GTP levels, improved adipogenesis. I also show that the exposure of 3T3-L1 pre-adipocytes to insulin can stimulate the association of Rab5 GEF via its VPS9 domain to the activated insulin receptor. This observation strongly suggests a direct link between the Rab5 inactivation and the insulin receptor trafficking and signaling.

Analysis of the Rab5-GTP levels reveals a significant decrease in the GTP levels in an insulin-sensitive manner during 3T3-L1 pre-adipocytes differentiation without any significant decrease of the total level of Rab5 (Figures 11). One striking feature of the Rab5-GTP levels in cells expressing Rab5 GEFs is that not only an increase of the GTP levels can be observed, but also a statistically significant decrease in the Rab5-GTP levels is displayed by comparing the Rab5-GTP levels between day 1 and day 9 during differentiation. In contrast, in cells expressing the Rab5 GAPs, the Rab5-GTP levels were not further affected by the addition of

insulin during the differentiation process. In this context, depletion of Rab5 GEFs or Rab5 GAPs by siRNA showed a remarkable characteristic; silencing of Rab5 GEFs decrease Rab5-GTP levels, while depletion of Rab5 GAPs increase it. These data suggest that regulation of these factors is critical to ensure and maintain the Rab5-GTP level during the differentiation process. Taken together, these results suggest a possible cellular mechanism of the regulation of Rab5 inactivation during adipogenesis, which is directly linked to the interaction of activated insulin receptor with the Rab5 GEFs.

It is reasonable to propose that association of activated insulin receptor with the Rab5 GEFs is specific and selective and will also affect the activation of Rab5. In support of this possibility, it has been shown that RIN1 interacts with activated insulin receptor via its SH2 domain (Hunker, Giambini, *et al.*, 2006). Interestingly, Rabex-5 and RIN1 are recruited by activated EGF-receptor tail via two different mechanisms: RIN1 require tyrosine phosphorylation of the EGF-receptor, while Rabex-5 requires ubiquitination. In addition, it has been also shown that a deletion mutant of Rabex-5 containing the VPS9 domain is targeted to membrane without Rabaptin-5 and the ubiquitination domain (Aikawa, 2012). Together, these data suggest that Rab5 GEFs utilize several mechanisms to interact with the activated receptor. However, in this study, I found that these Rab5 GEFs utilize one common domain (the VPS9 domain) to interact with the activated insulin receptor, with the exception of RIN1 that also utilizes the RIN1:SH2 domain to interact with the activated insulin receptor (Figure 34).

In addition, the VPS9 domain does not interact with insulin receptor catalytic inactive mutant or with other receptors (e.g., EGF-, transferrin- and LDL-receptor [data not shown]). However, the finding that the Rab5 GEFs VPS9 domain also interacts with the IGF-1R (data not shown) further support the idea that Rab5 GEFs VPS9 domain plays a critical function during the differentiation of pre-adipocytes since both the IR and IGF-1R have been implicated in the regulation of adipocyte differentiation and lipid accumulation *in vitro* (Boucher *et al.*, 2010). It is also important to mention that beside the VPS9 domain, other domains of the Rab5 GEFs may also play critical roles in the differentiation of pre-adipocytes. For example, the Ras-binding domain found in both RIN1 and Rabex-5, as well as the Ras-GAP domain described in RAP6, may also be involved in the differentiation of pre-adipocytes since the expression of Ras GTP-hydrolysis defective mutant (Ras:V12G) increases the differentiation of pre-adipocytes (Murholm *et al.*, 2010). These data suggest that Rab5 inactivation is a key and specific factor, yet not the only one, that is crucial to trigger the differentiation of 3T3-L1 pre-adipocytes.

In summary, my data show that the exposure of 3T3-L1 pre-adipocytes to insulin can stimulate the inactivation of Rab5, which strongly suggests a direct link between the inactivation of Rab5 and the activation of the insulin receptor. One explanation for this mechanism is lay on the association of Rab5 GEFs with the activated insulin receptor and IGF-1R. In either case, the VPS9 domain of Rab5 GEFs mediates this interaction with the activated receptor, indicating a novel cellular mechanism to regulate the activity of Rab5. This hypothesis is further supported by the observation that only tyrosine-phosphorylated insulin receptor

interacted with more than one Rab5 GEFs. Therefore, Rab5 cannot be activated once the Rab4 GEFs were sequestered by the activated IR or IGF-1R. In agreement with these observations, the addition of insulin down-regulates Rab5 activity in the initial phase, but Rab5 activity could be recovered later, which coincides with activation and inactivation phases of the insulin receptor during the receptor-mediated endocytosis. This lack of Rab5 activation will block insulin receptor internalization allowing an increase of the tyrosine autophosphorylation of the insulin receptor and activation of Akt followed by translocation of GLUT4 to the plasma membrane. Another possibility is that decreasing the level of Rab5-GTP is associated with diminishing the amount of Rab5 proteins during the differentiation process. Thus, inadequate level of Rab5 proteins would affect their activation by Rab5 GEFs. Moreover, it seems that it could be a case since the expression of Rab5 is differentially altered during the 3T3-L1 pre-adipocytes differentiation. However, it has been shown that the expression of Rab5 is not altered during the 3T3-L1 pre-adipocytes differentiation (Figure 22 and Figure 11). Additionally, secondary modification on Rab5 and/or Rab5 GEFs (e.g., phosphorylation, ubiquitination) would also be involved. Further investigation will be needed to define the potential requirements of specific modifications of Rab5. Additional explanations, such as how an increased GLUT4 internalization could be induced by an inhibitory effect of the Rab5 activity cannot be ruled out (Figure 13).

Here, I provided function and cellular evidence that Rab5 may be considered as a molecular switch on the cell fate determination of 3T3-L1 pre-adipocytes. Interestingly, Rab5 inactivation has been found to associate with

neurite outgrowth and differentiation, where RabGAP-5 interacts with NGF-TrkA receptor and regulates the NGF-TrkA receptor signaling (Tam *et al.*, 2015). Nevertheless, my studies indicate a finely orchestrated balance between GTP/GDP ratio of Rab5, Rab5 GEFs, and tyrosine autophosphorylation of the insulin receptor, and more importantly, demonstrate a fundamental role of Rab5 inactivation during the differentiation of 3T3-L1 pre-adipocytes.

III.6 References

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VITA

YONGJUN HAUNG

Education

2015 - present	Ph. D. Candidate Department of Chemistry and Biochemistry Florida International University, USA
2011 - 2012	NO.4 People's Hospital Wuxi, China Intern
2009	The First Affiliated Hospital of Soochow University Suzhou, China Professional practice
2008	Xiangyang Maternity and Child Health Care Hospital Xiangyang, China Professional practice
2008 - 2009	Leader of Research group of Anti-cancer Association Soochow University, China
2007 - 2012	BA in Clinical Medicine Department of Medicine Soochow University, China

Work Experience

2013 - 2015; 2017 - present	Teaching Assistant Department of Chemistry and Biochemistry; Department of Biological Sciences Florida International University, USA
2015 - 2017	Confocal Facility Assistant Manager Department of Biological Sciences Florida International University, USA

Publication

Birth of the adipocyte: Early adipogenesis, the MAPK pathway, and mitotic clonal expansion 2017

S. Abood, Y. Huang, M. A. Barbieri. *Current Topics in Biochemical Research* 18: 1 (2017)

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Y. Huang, M. L. Veisaga, V. S. Nadar, B. P. Rosen, and M. A. Barbieri.
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IGF-1 drives activation of Rab5 via Rin1 augmenting proliferation and invasion of MCF-7 cells 2018

N. Porther, Y. Huang, M. L. Veisaga, and M. A. Barbieri. *In preparation*

GTP hydrolysis of Rab5 controls differentiation of prediocytes 2018

Y. Huang, M. L. Veisaga, N. Villaverde, and M. A. Barbieri. *In preparation*

Oral Presentation

30th European Immunogenetics and Histocompatibility Complex May. 2016

S. K. Singh, Y. Huang, A. Caobi, M. A. Barbieri, M. T. Dorak. Functional Annotations of Common Disease Markers in Immune Regulatory Genes

19th Biology Research Symposium of FIU Feb. 2017

Y. Huang, M. L. Veisaga and M. A. Barbieri. Effects of Rin1 and its mutants on adipogenesis

2018 FIU Biomedical And Comparative Immunology Symposium Mar. 2018

Y. Huang. M. A. Barbieri. RIN1 Dictates the Fate of Fat Cell Formation

Poster

2015 ASCB Annual Meeting Dec. 2015

Y. Huang, E. Ocasio, M. L. Veisaga and M. A. Barbieri. A single amino acid change in Ras Interference 1 alters its function.

2016 ASCB Annual Meeting Dec. 2016

Y. Huang, W. Zhang, M. L. Veisaga and M. A. Barbieri. Selective effect of Rin1 mutants on insulin signaling during pre-adipocyte differentiation

2017 ASCB Annual Meeting Dec. 2017

Y. Huang, M. L. Veisaga and M. A. Barbieri. Role of Small GTPases on differentiation of pre-adipocyte

20th Biology Research Symposium of FIU Feb. 2018

Y. Huang, M. L. Veisaga and M. A. Barbieri. Small GTPases: friend or foe of adipogenesis?