Sequestosome 1 /p62 and TRAF6 serve as a bridge to connect IRS-1 with Akt in insulin signaling

by

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Abstract

Abnormalities in the insulin-signaling pathway might result in insulin resistance, contributing to the development of type 2 diabetes. Previous studies have established the involvement of p62 in the insulin-signaling pathway through its association with IRS-1 and the interaction of p62 with TRAF6. Studies conducted using L6 myotubes and the transfection of CHO/ IR cells indicated that IRS-1, p62, Akt and TRAF6 interact upon insulin stimulation. The interaction between p62 and Akt is impaired in TRAF6 knockout and p62 knockout Mouse Embryonic Fibroblast cells, confirming that TRAF6 connects p62 with Akt. Further, the transfection of ASp62 in CHO/ IR cells provided evidence that TRAF6 interacts with Akt but not with IRS-1 upon the reduction of p62 expression. In p62 knockout, IRS-1 does not interact with TRAF6 and Akt whereas in TRAF6 knockout, IRS-1 interacts with p62 but not Akt. Overall, these results demonstrate that p62 and TRAF6 link IRS-1 with Akt implying the existence of these proteins as a complex in the insulin signaling pathway.

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CHAPTER 1: INTRODUCTION

The seventh major cause of death in the United States, diabetes is "one of the most challenging health problems in the 21st century" according to the International Diabetes Federation (20, 19). This global epidemic is major reason for kidney failure, non traumatic lower-limb amputations, and new cases of blindness among adults in the United States (19, 20). Diabetes is also a leading cause of heart disease and stroke (19). Sedentary lifestyles, obesity and an aging population would lead to an elevation in the number of diabetic patients around the world to about 380 million by 2025 (16). In almost every country in the world, obesity and type 2 diabetes serve as a threat to the health of the population (14). Therefore, it is vital to examine the risk factors involved in this disease (16).

Type 1 diabetes occurs due to destruction of β-cells, usually resulting in complete insulin deficiency (18). A gradual defect in insulin secretion combined with insulin resistance leads to Type 2 diabetes (18). Other particular forms of diabetes may be a result of genetic defects in the functioning of β-cells or insulin action, exocrine pancreatic diseases such as cystic fibrosis and due to the effects of chemicals or drugs used to treat HIV/AIDS or post- organ transplantation (18). There is a steady elevation in the occurrence of type 2 diabetes in adolescents specifically among populations with ethnic minority (18). In 2010, about 215,000 people younger than 20 years were diabetic in the United States (19). In 2011, 8.2% or 4.6 million deaths across the world among the people between 20-79 years were due to diabetes (21). Increasing rates of childhood obesity among children has resulted in difficulty in distinguishing between type 1 and

type 2 diabetes (18). Ketosis and auto antigens might occur in majority of patients with characteristics of type 2 diabetes (18). Identifying a distinction between different types of diabetes is crucial during diagnosis as the treatment options, educational methods, and counseling for the diet of the patient is distinct (18).

A major complication among people suffering with Type 1 diabetes mellitus (T1DM) is hypoglycemia that is eventually even fatal to the patient (15). This effect is common among diabetic patients administered with insulin and is rare to develop among non-insulin-dependent diabetic patients who usually consume medicines that lower the glucose levels (15). Defects in normal secretion of insulin and elevated rates of insulin resistance lead to the pathophysiology of type 2 diabetes. (17). Type 2 diabetes has been characterized as a progressive disease where the net mass of the pancreatic cells decreases over time (17).

The action of insulin is defective in skeletal muscle, adipose tissue, liver, and the heart during insulin resistance and type 2 diabetes (43). The docking of insulin to its receptor initiates insulin action resulting in receptor tyrosine kinase phosphorylation and activation, further leading to the phosphorylation of many endogenous substrates, such as the insulin receptor substrate proteins (43). The IRS/phosphatidylinositol 3- (PI3) kinase pathway; (RAS)/mitogen-activated protein kinase (MAPK) pathway; and the Cbl-associated protein (CAP)/Cbl pathway are the three major pathways involved in the insulin signaling process (100, 126).

Insulin receptor substrate 1(IRS-1) is a vital player in the insulin signaling cascade (22). IRS-1 is involved in signal transmission to the intracellular pathways PI3K / Akt and Erk MAP kinase pathways from the insulin and insulin-like growth factor-1 (IGF-1) receptors (22, 24). Phosphorylation of the insulin receptors or IGF-1 receptors at the tyrosine residues, after the

docking of an extracellular ligand results in the stimulation of IRS-1 to these receptors in the cytoplasm, via the SH_2 domains (22, 24). The tyrosine phosphorylation of IRS-1 stimulates different signaling pathways, such as the PI3K pathway and the MAP kinase pathway (24). IRS-1 is involved in the biological functions of both metabolic and mitogenic pathways (24). In addition to a prominent impairment in growth, IRS-1 knockout mice also display a mild diabetic condition. Transgenic mice overexpressing IRS-1 suffered from breast cancer indicating the possible role of IRS-1 in cancer (24).

Sequestosome 1/p62 plays a role in receptor-mediated signal transduction and functions as a signal modulator or adaptor protein (323). Research involving p62 has gained prominance as it plays a role in the molecular mechanisms of different diseases such as Parkinson disease, Alzheimer disease, liver and breast cancer, Paget's disease of bone, obesity and insulin resistance (323). p62 knockout results in leptin resistance, which is also a characteristic feature associated with these metabolic disorders (276, 18).

An exception in the Tumor-necrosis factor (TNF) receptor-associated factor (TRAF) family to be involved in the signal transduction of both the TNF receptor (TNFR) super family and the interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily1–5, TRAF6 is crucial for adaptive immunity, innate immunity and also in maintaining the homeostasis in bone (241). TRAF6 associates with different protein kinases such as IRAK1/IRAK, SRC and PKC-zeta, which facilitates a connection between various signaling pathways (241).

The serine/threonine-specific protein kinase, Akt, also termed as Protein Kinase B (PKB), is involved in various cellular processes including glucose metabolism, apoptosis, transcription, cell migration and cell proliferation (34, 35). Phosphorylation of tyrosine residues of the insulin receptor substrate proteins results in the docking and stimulation of PI3K, leading to

phosphatidylinositol 3, 4, 5-trisphosphate formation and Akt activation (43). Akt has been connected in controlling glucose metabolism, cell growth, and anti apoptosis (320,191). Future research should focus on the characterization of the endogenous substrates mediating these responses (321). Originally observed as an oncogene in the transforming retrovirus, AKT8, Akt is a part of several signaling pathways including the PI3K/AKT/mTOR pathway (35).

Akt activation exerts its regulatory effects on various substrates including mTOR through its kinase activity (36). Akt1 stimulates protein synthesis pathways, and is the major signaling protein in the cellular pathways that result in skeletal muscle hypertrophy, and tissue growth in general (34). Due to its involvement in blocking apoptosis, Akt1 upregulates cell survival and is a crucial factor in different forms of cancer (199). Akt2 is essential for the translocation of glucose transporter 4 (GLUT4) to the plasma membrane upon insulin action (185). Glycogen synthase kinase 3 (GSK-3) could downregulated upon Akt phosphorylation, leading to an elevation in the synthesis of glycogen (36). GSK3 also plays a role in Wnt signaling cascade, in turn indicating the involvement of Akt in the Wnt pathway (36, 189).

The overall goal of this research was to examine the interactions of TRAF6, p62, AKT, IRS1 proteins in insulin signaling using cellular and molecular analysis. This understanding of different important components involved in the molecular mechanism of the insulin signaling pathway would further aid the research focusing on proteins to be targeted for the treatment of diabetes and its associated metabolic disorders.

OBJECTIVE AND HYPOTHESIS

Our first objective was to examine the association of TRAF6 with AKT and IRS-1. Our second goal was to analyze whether the interaction of p62 with AKT was through TRAF6. Finally, we sought to evaluate the role of TRAF6 in linking AKT to both p62 and IRS1. We hypothesized that the presence of both sequestrosome 1/p62 and TRAF6 was essential to link IRS-1 to Akt in the insulin signaling pathway.

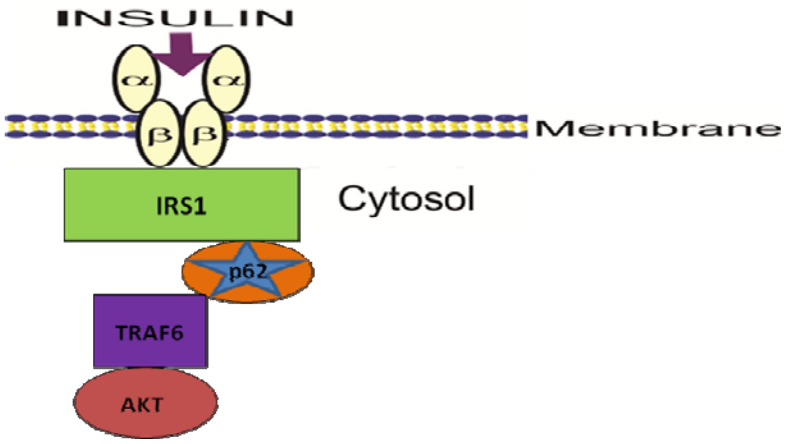

Figure 1: Model of the complex formed during insulin signaling. The interaction between these four proteins occurs only upon insulin stimulation. IRS1 interacts with TRAF6 through p62, while the interaction of Akt and TRAF6 occur independent of p62.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Diabetes:

2.1.1. Epidemiology

Distinct defects in insulin secretion and/or action lead to increased blood glucose levels causing not only a single disease entity, but a group of metabolic diseases known as diabetes mellitus (1). Long-term complications of diabetes result in the failure of major organs including the heart, kidneys, blood vessels and eyes (1). Heart disease, stroke is mainly caused due to diabetes. According to the National Diabetes Statistics diabetes has been ranked as the seventh leading cause of death in the United States (12). The pancreatic β-cells produce little or no insulin in the case of type 1 diabetes. Type 2 diabetes occurs due to insulin resistance, a condition wherein the body does not respond appropriately to insulin. Although the pancreatic cells produce more insulin in order to aid the uptake of glucose into the body cells, it lacks the ability to secrete enough insulin in response to meals (39). The disease in some patients is diagnosed as type 1.5 diabetes mellitus if the features of both type 1 and type 2 are observed (325). This form may be referred to as 'double diabetes'.

The twin-epidemics of obesity and diabetes are usually the consequences of uncontrolled eating habits. Considered to be a disease of the western lifestyle, obesity induces its related metabolic problems including insulin resistance and diabetes. In almost every country in the world, obesity and type 2 diabetes serve as a threat to the health of the population. 371 million people in the world are living with diabetes (17). The International Diabetes

Foundation has ranked China and India as the countries with the highest total number of cases of diabetes mellitus, with 90 million and 61 million cases respectively (2). Recent studies have predicted that by 2030, 79-87 million adults in India and 42-63 million adults in China will have diabetes (44). In the United States, it is currently estimated that over 25.8 million people or 8.3% of the population have diabetes (86). It is estimated that nearly 79 million people are currently living with pre-diabetes (86). According to the data collected by the Centers for Disease Control (CDC), the state of Alabama has the highest incidence of diabetes in the US after Mississippi (66). 11.1% of the people in Alabama had diabetes in 2010 (66). Currently, the prevalence of obesity in this state is 32.2% (88). According to the International Diabetes Federation (IDF), diabetes has been the cause for 4.8 million deaths worldwide in 2012, roughly 8.2% of total world mortality (17).

Gestational diabetes mellitus (GDM) is initially detected during pregnancy and is characterized by different levels of glucose intolerance (85). There is strong evidence to support the fact that there is a higher risk of abnormal glucose tolerance, obesity as well as metabolic syndrome in persons exposed to maternal diabetes *in utero* as children and young adults (326). Within 10-20 years after pregnancy, women who have been diagnosed with gestational diabetes have a 35-60% chance of developing type 2 diabetes. 2 to 10% of pregnancies have been reported with gestational diabetes (12).

2.1.2. Type 1 diabetes

Type 1 diabetes is an endocrine system disorder that is usually diagnosed in childhood. There is a lack in insulin production due to autoimmune destruction of the pancreatic β-cells. The invasion of a wide variety of leukocytes into the islet cells of the pancreas causes insulitis.

This stage, combined with prominent β-cell destruction in diabetes constitutes the two distinct phases of type 1 diabetes. The blood glucose levels are elevated during the second phase due to insufficient insulin production (9). The pancreatic β-cell destruction and the consequent type 1 diabetes have been thought to be triggered by various environmental factors such as certain viruses and dietary factors which in turn initiate the autoimmune process (10).

Before the time period when type 1 diabetes can be diagnosed, 80% of the β cells have already been destroyed in the pancreas. Occurring very commonly among a population of European origin, 2 million people in the Europe and North America are affected with type 1 diabetes (11). Complications during pregnancy, heart disease, damage to nerves and kidney, blindness are some serious outcomes of the disease. In the case of type 1 diabetes, insulin infusions or injections only act as life saving measures. The injections can neither aid in preventing diabetic complications or in curing the disease. As many as 3 million Americans have type 1 diabetes (12). Finland has the highest incidence of childhood type 1 diabetes in the world (89). The incidence of type 1 diabetes in children increases by 3% annually (12) and 78,000 children develop type 1 diabetes every year (92).

2.1.3. Type 2 diabetes

Irrespective of normal or even elevated insulin levels in the blood, defects in adipokine/inflammatory marker profile, insulin binding or/and insulin signal transduction lead to a failure in controlling the blood glucose levels (33). Almost every country in the world is suffering a threat to the health of its population due to Type 2 diabetes (307). According to the International Diabetes Federation, there were approximately 366 million people with type 2 diabetes mellitus in 2011, and this number is predicted to increase to 552 million by 2030 (2).

Childhood obesity is a critical cause for this significant increase (307). Obesity occurs with a BMI of 30 or higher and excess amount of body fat, causing heart disease, high blood pressure, and stroke. Nearly 1,11,909 excess deaths each year when compared to the normal weight category may be attributed to obesity related disorders (3). Until recently, only adults were affected with type 2 diabetes. The World Health Organization projected that globally, more than 40 million children under the age of five were overweight in 2010 (4). Now, an increasingly higher number of children are being diagnosed with obesity-related type 2 diabetes.

2.2 Insulin Action and Signaling

The regulation of carbohydrate, protein and lipid metabolism and maintenance of blood glucose levels are among the different biological responses activated by insulin (97). The amount of glucose produced by the liver is inhibited while the transport of glucose into muscle and adipose tissue are stimulated by sophisticated mechanisms and the coordinated role of insulin (43). The brain, the hypothalamus in particular has been shown to be involved in the maintenance glucose homeostasis (45-47). Patients with extreme cases of insulin resistance with mutations in the insulin receptor gene have shown to have a modified synthesis, degradation, and function of the receptor (48). The transmembrane heterotetrameric insulin receptor glycoprotein is made up of two α-subunits and two β-subunits. Insulin action begins with the binding of insulin to the extracellular α-subunit of its receptor. This binding acts as the stimulator to the tyrosine kinase activity which is intrinsic to the transmembrane β-subunit of the receptor (99).

The autophosphorylation of tyrosine residues on insulin binding is the first step of insulin receptor activation and the kinase domain at the juxtamembrane position undergoes a conformational change (49-50). This structural modification acts as an origin for the activation

of the kinase and its interaction with the downstream molecule that participates in the insulin signaling cascade. The phosphorylation of the insulin-receptor substrates (IRS) follows this autophosphorylation step (51-52). IRS contain a phosphotyrosine-binding (PTB) and/or the pleckstrin homology (PH) domains at the amino terminal, a COOH-terminal domain with tyrosine residues involved in Src homology 2 (SH_2) protein-binding sites, a Src homology 3 (SH3) domain that bind to proline-rich ligands and serine-threonine-rich regions which can further bind to other proteins. The insulin receptor is capable of phosphorylating many substrates on the Tyr residues including the IRS proteins (1-4), the three isoforms of Shc, Gab1, Cbl, APS, and p60dok that can inturn dock SH2 domain containing signaling proteins (53). These steps are followed by three main pathways, namely the IRS/phosphatidylinositol 3- (PI3) kinase pathway; (RAS)/mitogen-activated protein kinase (MAPK) pathway; and the Cbl-associated protein (CAP)/Cbl pathway that elicit proteins that are present downstream in the signaling system (102). Secondary messengers are produced by the phosphorylated PI substrates when the p85 regulatory subunit and p110 catalytic subunit of PI3 kinase (phosphoinositide 3-kinases) are activated. These messengers including the phospholipids Inositol phosphate [I(3)P], phosphatidylinositol bisphosphate [PI(3,4,5)P2] and phosphatidylinositol-trisphosphate (PIP3). The Akt (protein-serine/threonine kinase) and PI3K-dependent serine/threonine kinases (PDK-1) are involved in their movement from the cytoplasm to the plasma membrane. Akt undergoes structural modifications and is activated by PDK-1 upon its phosphorylation on Thr 308 and Ser 473 (54-55, 56). The glucose transporter 4 (GLUT4) complexes, glycogen synthase kinase (GSK3), the isoforms of protein kinase C (PKC) and p70 S6 kinase inturn get phosphorylated upon this Akt activation (54-59). Thus, by inactivating GSK-3, PI3K-Akt pathway regulates glycogen synthesis (60, 61). GLUT4

transport from intracellular sites to the plasma membrane, its activation occurs through insulin action and glucose uptake takes place in the muscle and fat tissue (Fig 2). Whole body GLUT4 homozygous knockout mice are associated with reduced lifespan, lower blood glucose levels and heart problems (62). Insulin resistance and glucose intolerance has been observed on GLUT4 disruption in the adipose tissue of mice as well as in muscle indicating the importance of GLUT4 to regulate blood glucose levels (63, 64). The Rab GTP-activating protein (GAP) domain of AS160, a 160kDa Akt substrate in 3T3-L1 adipocytes regulates glucose transport through the translocation of GLUT4 (103).

The second chief insulin signaling pathway plays the key role in cell survival processes including gene expression, DNA synthesis and occurs through the Grb2/Sos/Ras/Raf/MAPK (102). Other than the mitogenic signals, MAP kinase functional groups are also mediated by stress and inflammatory cytokines (102). SHC interacts with Grb2 upon phosphorylation and the MAPK pathway is activated irrespective of the presence or absence of IRS (102). The enzymes involved in gluconeogenesis including phosphoenol pyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) are selectively inhibited by insulin in the liver (67-69). This downregulation of the G6Pase gene transcription by insulin does not occur through the Ras/Raf/MAPK pathway but through the activation of the PI3 Kinase pathway in H411E hepatoma cells (69).

Tyrosine phosphorylation on C-Cbl upon insulin stimulation activates its binding to the insulin receptor through CAP (70). This step is followed by the movement of Cbl/CAP complex to the lipid raft subdomain of the plasma membrane (71, 73). Failure of the complex to translocate leads to the blockage in GLUT4 translocation irrespective of insulin stimulation (74). Also, the glucose uptake may fail to occur in 3T3-L1 adipocytes even upon

insulin stimulation, if the interaction between CAP/Cbl and the insulin receptor is blocked (102).Thus, the first and the third main insulin signaling pathways function parallel to each other and are compartmentalized in order to cause GLUT4 translocation.

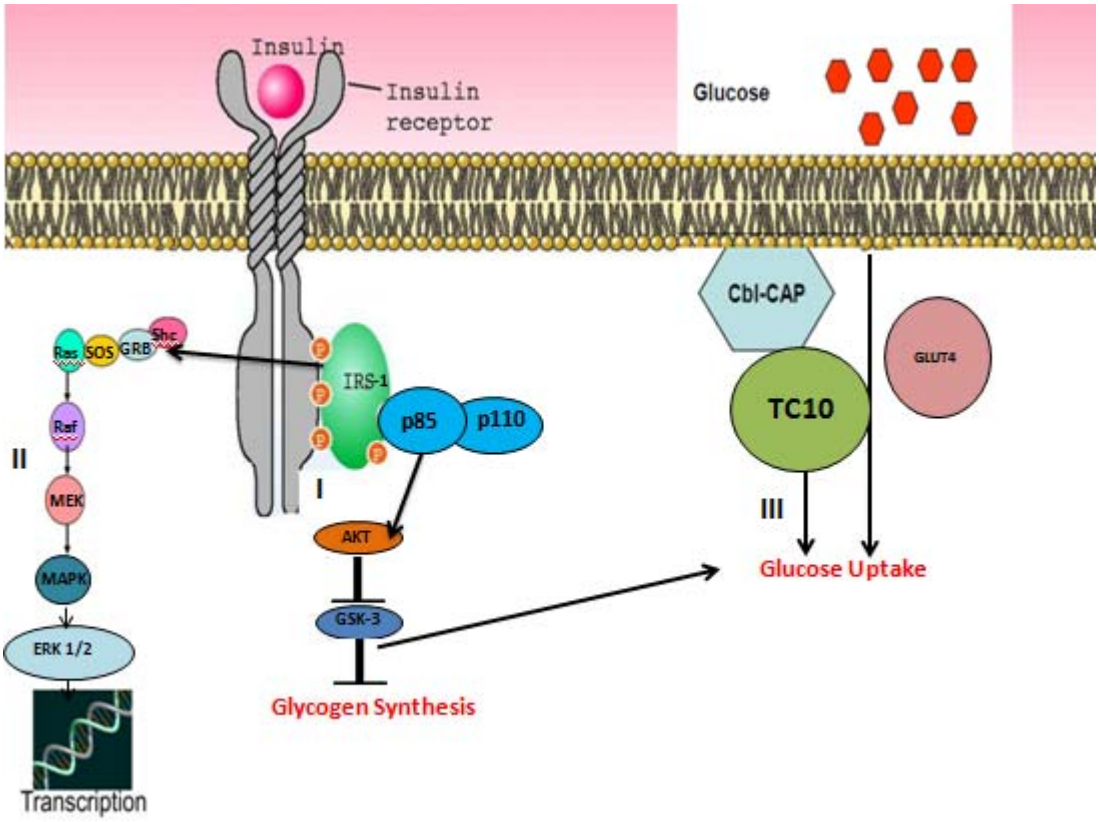

Figure 2: Insulin signal transduction (308, 309): Insulin is concentrated at the binding domain of the alpha-subunit of the insulin receptor. The alpha-subunit then undergoes conformational changes that further elicit the tyrosine activity of the beta subunit. This step further activated the downstream insulin signaling molecules into three main pathways.

2.3 Insulin Resistance:

The primary cause of type 2 diabetes is insulin resistance (327). Glucose intolerance and elevated blood glucose levels in case of insulin resistance are followed by a high blood pressure,

high lipid levels in the blood, heart diseases (syndrome X) and polycystic ovarian disease (PCOS) (327,42). The capacity of insulin to prevent the glucose output from the liver and to increase the uptake of glucose in fat, muscle is lost. This impairment in the cellular activities of insulin is termed as insulin resistance (105). The fat cells have increased resistance against the antilipolytic effect (36).

Lipid injection in muscle causes insulin resistance that increases the circulating Free Fatty Acid (FFA) levels (38). One of the important factors that control the insulin resistance of the entire body is abdominal fat accumulation (40). Women with PCOS commonly have insulin resistance (40). BMI of greater than 30 kg/m^2, low plasma levels of adiponectins, elevated albumin excretion rate in the urine, low HDL cholesterol, proinflammatory states, including thiazide diuretics, aging, adrenergic beta-receptor blockaders, smoking are usually the factors associated with insulin resistance (42, 19). Insulin sensitivity can be increased by weight loss, drugs such as metformin, thiazolidinediones (40).

2.3.1 Obesity

The most common and important risk factor for the development of type 2 diabetes mellitus (T2DM) is obesity. Data from surveys conducted by National Health and Nutrition Examination Survey (NHANES) in the year 2009-2010 showed that almost 17% of youth and more than one third of adults were obese (18). 78 million adults, 12.5 million children and youth in the US are obese (18). 15.5 million people are morbidly obese with obesity-related diseases such as type 2 diabetes, heart disease or sleep apnea (106). A body mass index (BMI) greater than 35 in men results in a 40-fold increase in the risk of T2DM, while a BMI greater than 35 in women leads to a 90-fold increase in the risk for T2DM (20).

White adipose tissue, representing the main form of adipose tissue expands and causes obesity (21).

This expansion takes place when the long term energy intake is greater than the energy spent by the body (21). Obesity is the most significant risk factor for the development of insulin resistance among children according to the NHANES data (23). Markers for inflammatory reactions in the blood include C-reactive protein (CRP), interlukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) (25-30). In case of obesity, the levels of these markers are elevated indicating a low grade systemic inflammation (25-30).

2.4 IRS-1

2.4.1: Different isoforms of IRS

Insulin receptors localize the binding of insulin in order to initiate its effects on the cellular growth and metabolism effects (90, 91). This step activates the insulin receptor Tyr kinase and causes the endogenous substrate phosphorylation (93). Tyr kinase activity is required for insulin action (110). The tightly regulated insulin receptor is a Tyr kinase, stimulated by insulin and mediated by a complex autophosphorylation cascade on Tyr residues (positive regulation) and on Ser and Thr residues (negative regulation) (107). The kinase is crucial for controlling most if not all of insulin's pleiotropic effects, as the receptors mutated at the ATP-binding site (Lys1030) fail to undergo phosphorylation on its own and its ability to act as a Tyr kinase is lost. Thus, the biological function of the receptor after the mutation is lost (108, 109).
Tyr kinase family has been divided into many classes. The epidermal growth factor (EGF) receptor are included under the class I receptors have a single unit with two cysteine rich motifs in the domain linked to the ligand (324). The insulin receptor and the IGF-1 receptors are structurally similar and are composed of cysteine-rich motifs in the α-subunit with disulfide

bonds in the tetramer (324). The receptors for the platelet-derived growth factor (PDGF) family, colony-stimulating factor-1 (CSF-1), c-kit and fibroblast growth factor, representing different single extracellular domain receptors with immunoglobulin-like units, are classified under the classes III and IV (324).

The number of insulin receptors differs among different mammalian tissues (94). The liver and the fat cells of the body have the highest concentration of the insulin receptor with more than 3,00,000 receptors per cell (94). Although the IGF-1 receptor concentration is elevated in skeletal muscles, the insulin receptor concentration is comparatively lesser (112). Insulin levels in the plasma are between 10^{-10} to 10^{-9}M, and are lower than binding affinity of insulin basal conditions (96,94). Hence, the cells that are specific targets for insulin have higher insulin receptors concentration to increase the kinetics of binding.

The insulin receptor gene on chromosome 19 is 150 kilobase (kb) and is composed of 22 exons and 21 introns (98-100). Disulfide bonds link the two α-subunits and two β-subunits covalently linked through the insulin receptor to form the $\alpha_2\beta_2$-holoreceptor (100). Estimates from nonreducing SDS-PAGE suggest that the molecular weight (M_r) of the heterotetramer ($\alpha_2\beta_2$) is 350,000 (100). According to the results from the reducing SDS-PAGE, the α-subunit has a M_r of about 135,000 and β-subunit about 90,000 (101). A ligand binding site is present in the α-subunit while the β-subunit is composed of an insulin-stimulated protein kinase (133).

The α-subunit of the insulin receptor is essential for ligand binding outside the cell (114). In general, the C-terminal domain of the α-subunit is composed of the alternatively spliced exon 11 (126). This domain functions as an important intermediate to transfer the signal from ligand binding to the insulin receptor kinase activation and is involved in a covalent interaction with the β subunit (128). A 23 amino acid domain, kinase targeting tyrosine residues present within the

cell, N- and O-related glycosylation site outside the cell are present in the β-subunit of the insulin receptor (104,100).

The first docking protein to be discovered, the insulin receptor substrate 1 (IRS-1) is represented as a model for this class of protein (133). Other proteins with similar properties associated with the IRS family include the IRS-2, IRS-3, Gab-1 and $p62^{dok}$ (113- 115, 133). IRS-1 and IRS-2 are involved in regulating the functions of IGF-1 and insulin in most tissues and IRS-3 is present in rodent adipose tissue, while the brain cells are rich in IRS-4 (134, 116, 117, 136).

Observations from modified mice have shown the different physiological functions of IRS-1. IRS-1 coordinates the development of body cells, while IRS2 combines the peripheral effects of insulin with β-cell function (119, 138). Mice models with IRS-2 knockout have shown to develop diabetes. IRS2 is also necessary for preventing the defects in CNS myelination and in murine photoreceptor cell maturation (123, 124). Studies from phosphorylated tau in the hippocampus portion of aged mice have showed the deterioration in brain development and neurofibrillary tangles formation after an IRS-2 gene knockout (122). Even after the disposal of IRS-3, growth and glucose regulation were normal unlike the degradation in growth in male mice and aberrations in the homeostasis of glucose that occur in IRS-4 knockout (120,121).

2.4.2. Structure of IRS-1:

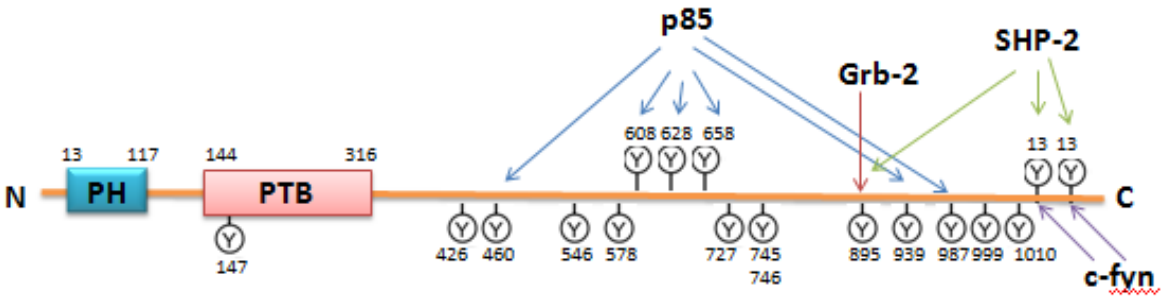

Figure 3: A representation of IRS-1 showing the various tyrosine residues; *PH*, pleckstrin homology domain; *PTB*, phosphotyrosine binding domain (310)

Chromosome 2q36-37 hosts the human IRS-1 gene that was initially discovered as a 185-kDa protein phosphorylated at the tyrosine residues (pp185) in hepatoma cells stimulated with insulin (127, 140). Originally cloned from mouse and human cDNA genomic libraries, there has been a high similarity in the sequences among different species of IRS-1 (125, 126). The absence of a kinase-like catalytic domain near its amino-terminus of IRS-1 unlike the common sequence for a nucleotide binding site indicates a different function of this region. IRS-1 also has a region present in many signaling molecules known as the pleckstrin homology (PH) domain and greater than 40 important sites for Ser/Thr phosphorylation which could act as substrates for casein kinase II, protein kinase A (PKA), protein kinase C (PKC) , cGMP-dependent protein kinases, MAP kinases, and cdc2 kinase (22, 129). Extensive serine phosphorylation takes place in IRS-1 compared to the lesser extent of Thr phosphorylation in the absence of insulin stimulation. There is a significantly higher rate of Tyr and Ser phosphorylation upon insulin stimulation (130).

The 20-22 important phosphorylation sites in the tyrosine residue which are preserved among the homologues of IRS-1 is one among its characteristic features (141). There is also a high degree of conservation between the amino acids around the homologues and many of these

serve as important binding sites SH_2 domain containing proteins (141). In animal studies, the phosphorylation of tyrosine residues by the insulin receptor includes the synthetic peptides derived from the IRS-1 sequence (131).

Modified IRS-1 phosphorylated with purified insulin receptors upon insulin-stimulation and phosphorylation in IRS-1 without insulin treatment was observed using tryptic phosphopeptide analysis of baculovirus. From this analysis, a minimum of at least eight important sites of Tyr phosphorylation at 460, 608, 628, 895, 939, 987, 1172 and 1222 have been observed (132). IRS-1 also binds to the IGF-I receptor as a substrate in both in cultured cells and *in vivo* (303. 133).

The phosphorylation of IRS-1 by the IGF-I receptor takes place at similar positions as observed by tryptic phosphopeptide mapping and, in addition, stimulates the association and activation of PI3-kinase (141). The purification of PDGF, CSF-1 or EGF receptors has not lead to IRS-1 phosphorylation (141). Thus, eventhough IRS-1 is involved in both IGF-1 and insulin signalling, other Tyr kinases receptors do not utilize IRS-1 (141).

The interaction between PI3-kinase and the protein phosphorylated at tyrosine residues was initially observed in the polyoma middle T antigen (304). This interaction demonstrated in the activated PDGF receptor happens through the SH_2 domains. It has been shown in particular phosphorylated motifs containing the homologous peptide motifs YMXM or YVXM sequence (where X is amino acid, Y is tyrosine, V is valine, and M is methionine). Nine of the fourteen vital phosphorylation sites of tyrosine residues in IRS-1 occur in these two sequences (22). The kinase gets activated due to the binding of PI3-kinase with IRS-1, the addition of YXXM peptides upon phosphorylation or IRS-1 phosphorylation *in vitro* (142).

PI3-kinase contains the SH_2 domains which specifically bind to four of the phosphopeptides obtained from IRS-1 sequence around Tyr 460, 608, 939, and 987 (132). Insulin signal transmission is phosphorylated by IRS-1 that functions as the "docking" protein and docks, controls the enzymes with the SH_2 domains present within the cell (141). The 22 sites of phosphorylated Tyr residues in IRS-1 serve as possible sites to bind the proteins containing the SH_2 domain (141).

Other than the p85 subunit of PI3-kinase, GRB-2 and three different SH2 proteins have been shown to interact with IRS-1 (141). p85 has two different isoforms, namely p85α and p85β (305). Consensus sequences occur in both the isoforms which interact with IRS-1 phosphorylated at its Tyr residues (141) and with a p110 catalytic subunit of PI3-kinase (305,137). The sequences that occur in the two isoforms differ significantly and are responsible for their distinct signaling abilities (141).

Tyr phosphatase Syp (SHPTP2) protein includes two SH2 domains that docks IRS-1 at the position 1172 of the tyrosine receptor (132, 139, 302). The Syp/IRS-1 complex synthesis not only provides another possible signaling involving IRS-1, but is also a mechanism for downregulating the signal obtained from insulin stimulation (141). Nck is a 47-kDa adapter protein consisting of the three SH3 domains and one SH2 domain which connects IRS-1 to the PDGF and EGF receptors (Tyr kinases present upstream) and to other downstream pathways involved in cell growth (144, 149). The control of Tyr and Ser/Thr phosphorylation demonstrates a different degree of regulation in the signaling pathway of insulin, *in vivo* (141). IRS-1 interacts with different subgroups of the SH2-containing proteins. This plays an important role in order to make IRS-1 as a vital signaling molecule.

2.4.3 Functional Properties of IRS-1

An excellent candidate IRS-1 for differential regulation during different stages of diabetes, IRS-1 acts as an important substrate for the insulin receptor. Studies have been conducted to observe the regulation of IRS-1 and its phosphorylation by employing IRS-1 antibodies in tissues sensitive to insulin stimulation (muscle and liver) under both fasting and fed states. These investigations used control animals, streptozotocin (STZ)-induced diabetics, mouse model of type II diabetes with genetically modified insulin resistance and ob/ob mouse (150). After fasting, the activity of the insulin receptor increases both in the liver and muscle. This upregulation was accompanied by higher rates of insulin-stimulated receptor phosphorylation along with an increase in the phosphorylation of IRS-1. Under similar conditions, the phosphorylation of IRS-1 increased by ~2 fold (150). The IRS-1 levels in the muscle were inversely proportional to the duration of fasting unlike the IRS-1 levels in the liver. (150).

In vitro data on cultured adipocytes and CHO cells expressing insulin receptors with erbB-2 oncogene as well as *in vivo* data from ob/ob insulin-resistant mouse model of type II diabetes with high levels of insulin in the blood and from dexamethasone-treated rats demonstrated a downregulation in IRS-1 phosphorylation implying the crucial roles of the protein in insulin-resistant states (158, 173, 148, 193). 4PS occurring in myeloid cell lines is similar to IRS-1 and is involved in the phosphorylation of tyrosine residues (148). Upon insulin and interleukin-4 stimulation, 4PS produces a positive effect on cell growth (148).

In case of STZ-diabetic rats, an increase in the rates of IRS-1 phosphorylation indicates elevated number of receptors and increased activity in both liver and muscle (150). These results are comparable to the reduction in the activity of insulin receptor Tyr kinases known to occur in

STZ diabetes (151). During the fasting state, the tissue-specific control of IRS-1 is lower in muscle when compared to the high rates of regulation exerted in the liver (150).

Angiotension II (ANGII) has been associated with insulin resistance. Many randomized, clinical trials have recorded in vascular abnormalities with diabetes an improvement angiotension-converting enzyme inhibitors (ACE-I) and ANG II type I receptor blockers (ARBs) administration (61). Mutations in 3-phosphoinositide-dependent kinase-1 (PDK1) in the absence of Tyr phosphorylation downregulates the degradation of IRS-1 as well as the phosphorylation of IRS-1 on Ser307 (61). PDK1, the serine/threonine kinase dependent on phosphoinositol-3-kinase (PI3K) activates Akt and stimulates the movement of glucose transporter (GLUT-4) to the cell membrane (61). Ang II serves as stimuli to elicit C-Jun NH2-terminal kinase (JNK), which belongs to a family of mitogen-activated protein kinases (146). IRS-1 phosphorylation at Ser 308 occurs due to the interaction of JNK with IRS-1 in CHO cells through the JNK-binding sites in the carboxyl terminus of IRS-1 (146). A reduction in activity of JNK in human umbilical vein endothelial cells by insulin-induced production of nitric oxide reverses the downregulatory effects of ANG II (147).

The three important types of cell lines employed to study the part of IRS-1 during insulin action employ Chinese hamster ovary cells (CHO cells) overexpressing the insulin receptor (CHO/IR), IRS-1 (CHO/IRS-1), or both insulin receptor and IRS-1 (CHO/IR/IRS-1) (130). Without insulin stimulation, Ser residues are phosphorylated at higher rates than Thr residues in all the three cell lines (130). Compared to untransfected CHO cells, insulin increases the IRS-1 phosphorylation at Tyr residues by about two-fold in CHO/IR and CHO/IRS-1 cells and approximately fifteen-fold in CHO/IR/IRS-1 cells (130). IRS-1 expression matches with greater level of insulin-response in the cells corresponding to an interaction with PI-3-kinase (130). The

sensitivity of PI3-kinase activity upon insulin stimulation is high when the CHO/IR/IRS-1 cell lines are used as the overexpression of the insulin receptor and IRS-1 improves sensitivity and responsiveness while when the use of CHO/IR cell line increases the maximal response of PI3-kinase specifically (130). DNA synthesis in CHO/IR cells is more sensitive and CHO/IRS-1 cells are more responsive to insulin upon insulin-stimulation (130).

2.5 Sequestosome 1/p62

2.5.1: Structure of p62

Figure 4: A model of full length p62: AID, acidic interaction domain; LIR, LC3-interacting region (310).

The adaptor protein, sequestosome 1/p62 acts as an intracellular signal modulator in receptor-mediated signal transduction (298). p62 is composed of many structural motifs which are involved in multimeric signaling complex formation (278). The N-terminus has the SH_2 binding domain inside the ubiquitin-like domain (UBL) and an acidic interaction domain (AID/ORCA/PC/PBI) that docks the PKCζ (278, 279). In the carboxyl terminus, the ZZ finger, a site for the RING finger protein tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) binding, the ubiquitin-associated (UBA) domain and two peptide sequences rich in proline, glutamic acid, serine, and threonine (PEST sequences) (278). p62 has an array of

regions that are associated with related signaling proteins. The N-terminal AID (OPCA)-containing PB1 region, of p62 associates with the uniform homologous PB1 domain of the PKCζ, while TRAF6 through a comparatively small stretch of amino acids located in the central part of the protein (272). p62 plays a role in shuttling the polyubiquitinated substrates for proteosomal degradation (298). The proteasome interacts with the PBI domain at the amino terminus of p62 while K63-polyubiquitinated substrates associate with the C-terminal UBA domain (amino acids 386 to 440), that during defects in the proteasome, form aggregates to compromise cell survival (298). Several variations of p62 exist after splicing, including absence of the C-terminal UBA domain or one without the TRAF6 binding site (277, 298). Hence, different complexes may occur *in vivo* and exist in a tissue- as well as context -specific manner.

2.5.2 p62 family proteins

Human p62 plays a role in several biological processes. p62 was cloned by two different research groups as the ZIP for PKCζ-interacting protein as it cointeracts with the atypical protein kinase Cζ (PKCζ) (258). p62 was first observed as a ligand independent of phosphotyrosine in the Src homology 2 (SH_2) domain of $p56^{lck}$ (259).

The transcription of the intermediate-early response gene that generates the p62 protein is regulated by several extracellular signals that are involved in differentiation, cellular proliferation and cellular reactions to oxidative stress, immune response (260-262). Homologues to human p62 (263), have been found with upto 90% sequence homology and binding domains, that are conserved occur in other species such as rat (ZIP)(264) and mouse (A170/STAP) (265, 266).

2.5.3 Functional properties of p62

p62 and its homologues are composed of many structural motifs that regulate its association with polyubiquitin and various signal transduction proteins. p62 may also be involved in tumor necrosis factor alpha (TNFα), interleukin 1 (IL-1), nerve growth factor (NGF) and K+ channel activated signal transduction by 'adapter' protein interaction with signaling proteins located upstream (288, 294, 270). p62 also connects them to the common downstream component, PKCζ, thus stimulating the activation of nuclear transcription factor κB (NFκB). p62 might play a role in mediating gene transcription in the nucleus by association with the PKCζ (267-269). p62 acts as a scaffold protein by indicating PKCζ in specific signaling pathways (270), leading to the selectivity of these kinases (271).

p62 affects many of the biological effects of insulin by participating in three distinct signaling cascades. Insulin binds to the insulin receptor (IR) at the plasma membrane eliciting the insulin-related signaling in β cells (138). This causes the stimulation of PI3-kinase and protein kinase B (PKB/Akt) through the phosphorylation of Tyr residues in the insulin receptor substrates (IRS) (290).

The activation of NFκB may also play a role in insulin signaling. PKCζ regulates this signaling process (291). The activation of IL-1 or TNFα-elicited signaling in β cells can also lead to NFκB stimulation (294). p62 plays a role as an adapter protein in these signaling cascades by serving as a connection between TRAF6 and receptor interating protein (RIP) present upstream to the downstream effector, PKCζ (292).

2.5.4. Physiological function of p62 relevant to human diseases

The obesity and insulin resistance of mice increases when the p62 KO colony begins to age (276). These observations indicate that the absence of p62 causes mature-onset obesity, insulin and leptin resistance. The amount of energy spent by p62-deficient mice also changes

(276). Biochemical analysis of these mice suggests that this loss of p62 upregulates the basal ERK activity that is necessary for regulating adipocyte differentiation (276). Deletion of p62 also leads to leptin resistance, another feature associated with these metabolic disorders (276, 18).

Noncovalent interaction occurs between ubiquitin and p62 (279). p62 is a vital part of intracellular cytoplasmic aggregates composed of ubiquitinated proteins, which are the characteristic features in a several long term degenerative metabolic or neoplastic, toxic disorders including alcoholic hepatitis, hepatocellular carcinoma, α1-antitrypsin deficiency, Alzheimer's disease, Parkinson's disease, amylotropic lateral sclerosis and astrocytoma (280-286, 223). In particular, hallmarks such as the Lewy bodies in the case of Parkinson's disease, Mallory bodies in steatohepatitis and neurofibrillary tangles in Alzheimer's disease are examples (285, 323). Further research is needed to prove the involvement of p62 in the production of protein aggregates. The association of p62 with the aggregates might determine its stability as well as its roles on cellular biology (286).

TRAF6 plays an important role in regulating osteoclastogenesis and bone remodeling *in vivo* (273). The absence of p62 causes aberrations in osteoclastogenesis when mice are treated with the calciotropic hormone PTHrP, and bone marrow osteoclast precursors are challenged with RANK-L *in vitro* (274). However, this loss of p62 does not cause important changes in the basal physiology of bones (274). Similar to the effects of p62 in osteoclast differentiation, after the maturation of $p62^{-/-}$ mice, the bone density is elevated significantly (274).

Unlike the healthy tissue, p62 is colocalized with Aβ peptide, ubiquitin, tau in the tangle structures from the brain samples of Alzheimer's disease (AD) patients (286, 223). When there are defects in the normal functioning of the proteosome, TRAF6-K63-polyubiquitinated

substrates are taken up by p62 aggregates leading to the neurodegenerative disease process (298). 5q35-linked Paget's disease of bone is caused due to mutations in p62's UBA domain and aberrant osteoclastic activity, neurological complications, higher risk of fracture and osteosarcoma are associated with this defect (273). Samples obtained from patients suffering from this disorder have proved the presence of a point mutation in the P3921 premature stop codon, leading to a translation with the absence of the UBA domain or the splice site being mutated. Hence, the inability of particular polyubiquitinated substrates to associate with the UBA domain of p62 might characterize the pathophysiology of Paget's disease (273, 323).

Unlike the diseases where the involvement of p62 in the cytoplasmic protein aggregates could be detected by antibodies, the expression of p62 in normal tissues has not been studied (282). The interaction of p62 with cytoplasmic inclusions is absent when p62 is diffused in the cytoplasm of pancreatic β cells obtained from a healthy tissue (283, 281). The expression of p62 along with insulin demonstrated the role of p62 in β cell- specific and insulin-related signal transduction (289, 283).

The role of p62 in IL-1 and TNFα-signalling pathways can alter some of the effects of NFκB-dependent effects, such apoptosis (293). The activation of the serine/ threonine kinase PKCζ in non-β cells, by TNFα may be involved in physiological negative insulin feedback regulation as well as insulin resistance, since it mediates phosphorylation of serine residues of IRS proteins (293). p62 might be involved in these processes. The expression of p62 is reduced to a great extend or even destroyed in neuroendocrine tumors of the pancreas, that can reduce the sensitivity of tumor cells to NFκB-mediated pro-apoptotic signals (294). The association of p62 with TRAF6 plays a vital role in inflammation, cell survival and tumorigenesis (294). Many

studies have observed the importance of regulating the levels of p62 in order to prevent cell toxicity and cancer.

2.6. TRAF6

2.6.1 Structure of TRAF6

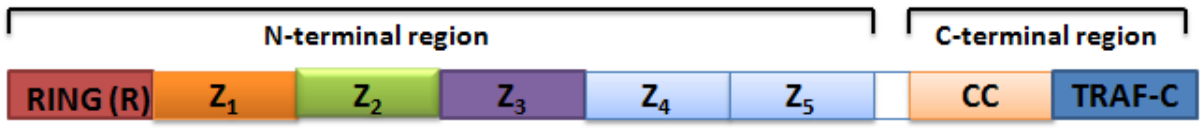

Figure 5: Domain organization of TRAF6. Z_1–Z_5, zinc fingers 1–5; CC, coiled coil (311).

The family of TNF-receptor associated factors (TRAFs) were first observed among humans and rodents for their ability to associate with several members of the TNFR family (207). Seven TRAFs occur in humans and other mammals (TRAF1 to 7). TRAFs contain a C-terminal region (TRAF-C domain), are composed of about 180 amino acids (300). Meprins are a part of a family of extracellular metalloproteases (299), also containing the characteristic C-terminal domain which has a very similar sequence homology as the TRAF-C domain and thus capable of forming similar anti-parallel β-sheets fold (210). Hence, the TRAF-C domain is termed as the meprin and TRAF Homology domain (MATHd) (255). Crystallographic studies on human TRAF2 and TRAF3, the MATHd have recorded the presence of a coiled coil TRAF-N domain followed by a 7-8 anti-parallel β-sheet fold (211). Further, these studies have also indicated the interaction of mammalian TRAFs in trimers, with the coiled coils intertwined to aid in the stability of the complex (211), the membrane-organizing protein caveolin-1 (226), the microtubule-binding protein MIP-T3 (224) and filamin (225). Other than TRAF1, the TRAF family-members are also composed of distinct zinc finger domains preceded by an unique N-terminal RING finger domain (212, 213).

Although the TRAF domain is absent in TRAF7, it still is classified under the TRAF family because it is composed of zinc finger domains and a RING finger domain present in the TRAF family members and also since it might be involved in the regulation of TNF-family signaling similar to the other TRAFs (215, 207). Like the other candidates under the MATHd superfamily, TRAFs seem to participate in proteolysis. TRAF2 might act as an E3 ubiquiting ligase that control TRAF3 and TRAF2 ubiquitination, proteolysis (216, 217). TRAF3 might mediate NIK proteolysis (218, 207).

TRAFs (specifically TRAF2 and TRAF6), together with the E2 ligase complex Ubc13/Uev1A, are involved in the ubiquitination of other proteins with K63-linked poly-ubiquitin chains (219). Unlike K48 ubiquitination which specifically targets TRAFs or other proteins for degradation, K63 ubiquitination leads to the involvement of TRAF2 and TRAF6 to elicit other components of the pathway (219).

The structure of TRAF6 domain is closely related to that of the other TRAF family members (255). Five zinc finger regions, a RING followed by a coiled-coil TRAF-N domain and a conserved TRAF-C domain are present in the amino terminus (233). Self-association and receptor interaction are mediated by the carboxy terminus, while the amino terminus of TRAF6 regulates downstream signaling (234). Mapping studies have proved that the residues 351-522 in the TRAF-C domain lead to its association with the receptor (235, 236). At elevated TRAF6 concentrations, TRAF6 occurs as a trimer, similar to the structure of TRAF2 (237).

The TRAF-C domain of TRAF6 demonstrates a greater level of variation than other TRAF protein structures and when compared to the TRAF2 TRAF-C domain (241, 255). Many residue deletions or insertions exist among the loop regions in the structure of TRAF6 (207). Proline residue insertion in the β-bulge of the β7 strand (P468) causes an important structural

distinction between TRAF6 and TRAF2 (207). The insertion of this proline residue also causes the formation of excessive main chain hydrogen bonds between the TRAF-C domain and the receptor peptides (207). The distinct surface pocket for the P-2 residue is a vital factor for deciding the variety in peptide binding by TRAF6 relative to TRAF2 (255). Phe 471 and Tyr 473 replace Ser 467 and Cys 469 in the P-2 pocket in TRAF2. This aids in the formation of another pocket for the P-2 proline residue present about 3Å from that in TRAF2 (255).

Hydrogen bonds stabilize the amide nitrogen atoms of Leu 457 and Ala 458 present in the main chain with the carboxylate of the P_0 glutamic acid residue (241). The aliphatic region of the side chain occurs near the TRAF6 surface (241). Tyr 349 of the P3 residue or Phe 238 of CD40 of RANK is present next to many aromatic and basic residues of TRAF6 (241). Arg 392 of TRAF6 and Tyr 349 of RANK are connected through amino-aromatic linkages (241).

2.6.2 Functional properties of TRAF6

The TRAFs are important for dorsoventral polarization and innate host defense through signal transduction elicited via the Toll receptors in Drosophila (207). TRAFs are the essential factors in signal transduction of the toll-like receptor (TLR) family and the tumor necrosis receptor (TNFR) family (255, 207). The binding of effector proteins and kinases to stimulated receptor and other signaling complexes is mediated by the TRAFs (220, 221). TRAFs have been associated with proteins of the cytoskeleton and/or of specific membranes such as p62 nucleoporin, a component of the nuclear plug (207).

TRAFs also regulate the stimulation of components present downstream in these pathways, to mediate the level of the response and modulate the subcellular relocalization of the receptor-ligand complexes by mediating the degradation of chief proteins in the pathway (255). The oldest among the mammalian TRAF proteins, TRAF6 was initially observed independently

by yeast two-hybrid screening with the cytoplasmic tail of CD40 as bait (227) and screening of an expressed sequence tag (EST) (226). TRAF6 has distinct TRAF-C domain when compared to the other members of the TRAF family and interacts with the cytoplasmic tails of receptors and other upstream molecules (255). The amino acid sequences identified by the TRAF-C domain vary from those recognized by other TRAFs (255). While TRAF2 and TRAF5 are associated with the P-X-Q-X-T motif, TRAF6 interacts with the X-X-P-X-E-X-X-Acidic or Aromatic consensus-binding site (228).

TRAF6 has different physiological functions when compared to other TRAFs due to this difference. The Src family nonreceptor tyrosine kinases including c-Src can be stimulated by TRAF6 (229). The affinity of TRAF6 is lower towards other monomeric receptors as the recruitment of TRAF6 depends on increasing the affinity through receptor and adapter protein oligomerization (230). Unlike the other TRAF family members, TRAF6 has distinct sequence specificity for receptor binding (231, 232).

Initially observed in the signal transduction pathways of CD40 and IL-IR (241, 242), the crucial biological role of TRAF6 in IL-1R/TLR signaling has been identified by the specific deletion of TRAF6 (239, 240). Among the members of the TRAF family of adaptor proteins, TRAF6 is the only member to regulate signals from both the TNFR and the IL-1R/TLR superfamily (272, 255). Studies on the gene deletion of TRAF6 have stressed the involvement of TRAF6 in innate and adaptive immunity, bone metabolism, the development of lymph nodes, mammary glands, skin, and the central nervous system (243-245).

TRAF6 is also involved in the signal transduction of many receptors, pathogens and pathogenic proteins other than the TNFR and IL-1R/TLR superfamily (255). The association of the Epstein-Barr virus transforming protein, LMP1 signaling with the p38 MAP kinase is mainly

disrupted in TRAF6-knockout fibroblasts (246). Other than the role of TRAF6 in inflammation and cell survival, TRAF6 knockout causes a higher degree of neural-tube-closure failure and exencephaly (247).

Studies with TRAF6-deficient mice have indicated the vital role played by TRAF6 in various critical processes which cannot compensated by the other TRAFs (248, 272). Exogenous TRAF6 expression in $TRAF^{-/-}$ Mouse Embryonic Fibroblast (MEF) cells restores the stimulation of nuclear factor kappa-B (NFκB), jun-amino terminal kinases (JNK), and p38 upon Interleukin-1 (IL-1) treatment (272). TRAF6 is essential for IL-1 signaling linked to NFκB and MAPK activation (248, 272). TRAF6 is essential for the Toll/IL-IR (TIR) domain-containing adaptor protein, MyD88-dependent pathway (249, 248).

Dendritic cells (DCs) are involved in acquired immunity by capturing, processing, and presenting antigens to T cells (250). $TRAF6^{-/-}$ bone marrow chimeras indicated a significant reduction in the occurrence of DCs, suggesting the importance of TRAF6 *in vivo* (250). Unlike Interleukin-7 Receptor α^{+} (IL-7Rα^{+}) cells from $TRAF6^{-/-}$ embryos, the cells from control embryos express lymphotoxin α1β2 (Lα1β2) when stimulated by the receptor activator of nuclear factor kappa-B ligand (RANKL). This suggests that the RANK-TRAF6 pathway is important for Lα1β2 expression in cells expressing IL-7Rα during lymph node organogenesis (251, 255). The presence of thymic microenvironments is vital for the normal development of regT (T-cells) that occurs through TRAF6 and NFκB-induced kinase (NIK)-mediated signals (251, 255). TRAF6 acts as the molecular connection between innate and adaptive immunity (255).

The association of RANK with TRAF6 is important for the normal reabsorption and for the efficient cytoskeletal structure formation (252, 255). $TRAF6^{-/-}$ mice display defective

osteoclast function or the total loss of osteoclasts (255). TRAF6 is necessary as a signal transducer for X-linked ectodysplasin-A2 receptor (XEDAR) (244). TRAF6 associates with XEDAR, ectodysplasin receptor (EDAR) (255) and tumor necrosis factor receptor superfamily 19 (TNFRSF19), which belong to the TNFR superfamily, in the formation of epithelial appendices (254). The neutrophin receptor p75 is classified under the TNF superfamily. p75 causes apoptosis after neutrophin activation or increases survival in accordance with its concentration in the cell (238). p75 signaling analysis in mice indicate the crucial role played by TRAF6 in the pathway (238).

TRAF6 is involved in protein translocation in response to nerve growth factor (NGF) (257). Without the presence of TRAF6, the translocation of neutrophin receptor interacting factor (NRIF) to the nucleus is prevented (256). Nerve growth factor (NGF) stimulates TrkA polyubiquitination and the p75 receptor (257). When the TRAF6-mediated polyubiquitination of TrkA is blocked, the receptor is retained at the membrane (257). TRAF6 is necessary for NGF-dependent internalization of TrkA and signaling (256).

As TRAF6 plays a role in inflammation and tumorigenesis, reducing the effects of TRAFs may be useful in the therapy of several diseases (306). By preventing the association of TRAF-receptor with short peptides or small molecules, TRAF6 signaling could be inhibited (306). Questions related to the form of TRAF6 causing the conformational changes leading to its stimulation and the involvement of ubiquitination in the activation of TRAF6 remain to be elucidated. Owing to its role as a common mediator for several distinct signals occurring both upstream and downstream, TRAF6 will remain as a vital focus of examination for diverse biological interests.

2.7 AKT

Conserved from primitive metazoans to humans, Akt (also known as Protein Kinase B, PKB) is classified under the subfamily of the protein kinase superfamily, and is composed of 518 members in humans (152). After a decade from the first observation of transforming murine leukemia virus Akt8 as an oncogene, Akt was initially cloned in 1991 by three different groups (153, 154).

2.7.1 Isoforms of Akt

In mammals, the Akt subfamily consists of three important isoforms, Akt1, Akt2 and Akt3 (61). All the three isoforms have almost identical specificity for the substrate *in vitro* and share a great level of amino acid identity (61). But *in vivo*, these isoforms have preferred substrates. This might be because of the varied cellular localization of the isoforms that decides their association with a specific various group of target proteins. In various mammalian tissues, the comparative expression of the isoforms vary: Akt1 is the major isoform in most of the tissues (157), Akt2 is predominant in adipose tissue, liver and skeletal muscle which are the organs and tissues that are more responsive to insulin and the isoform Akt3 exists abundantly in brain and testes (157, 191).

Akt1 is involved in the regulation of placental development and maintenance (61). Akt1 null mice have elevated rates of apoptosis in thymus and testes accompanied by mild growth retardation (61). Akt2 null mice express a severity in insulin resistance in accordance with the strain's background (157). High levels of glucose, insulin in the blood and glucose intolerance are caused as the insulin action is affected due to the absence of Akt2 (157). Akt2 is mandatory for normal insulin action on GSK-3α in cultured adipocytes (157, 191). Akt2 null mice also demonstrate a progressive β-cell failure, mild growth retardation and an age-dependent loss of adipose tissue (lipoatrophy) (191). Thus, these results indicate that Akt2 has an important part in

glucose metabolism, adipogenesis and maintenance, β-cell function and in the normal growth of the animal (191). The brain size of Akt3 null mice is about 20% smaller suggesting that Akt3 is needed for maintaining the normal brain size (191).

Although the Akt1/ Akt2 double-knockout (DKO) mice are born alive, they die immediately after birth, mainly due to an inability to breathe caused by the atrophied diaphragm muscles (159). Owing to the significant reduction in the individual muscle cell size, these DKO newborns are about 50% smaller that WT littermates, and are affected with defects in skin and bone development with extreme skeletal muscle atrophy (159). Additionally, total ablation of adipogenesis is observed in the Akt1/Akt2 DKO neonates (159). During the conditions when insulin-stimulated glucose uptake is replaced by the partial loss of Akt2, Akt1 is necessary for half of the remaining insulin signal (157, 161). Progressive loss of Akt1, Akt2, or both causes a correspondingly progressive reduction in glucose transport stimulation (160, 157). The defects recorded in Akt1/Akt2 DKO mice phenocopy IGF-1 receptor or insulin receptor-deficient mice, indicate that Akt performs the most crucial functions of the IGF-1 receptor and/or insulin receptor during development (156, 157 162). $Akt1^{+/-}Akt2^{-/-}Akt3^{-/-}$ mice are born alive, survive despite of being severely diabetic and are about 50% smaller when compared to wild-type mice (161, 162, 157).

Research thus far on Akt-deficient mice suggest that one allele of Akt1 is enough to assist embryonic development and adult survival, while Akt2 cannot support embryonic development on its own (163). Further, Akt3 can aid embryonic development by itself but not adult viability (163). The removal of these Akt isoforms mainly depletes insulin signaling in both GLUT4 glucose transporters and glycogen synthase kinase (GSK)-3, indicating a mandatory role of the Akt protein kinases in the insulin-signalling cascades (157).

2.7.2 Structure of Akt

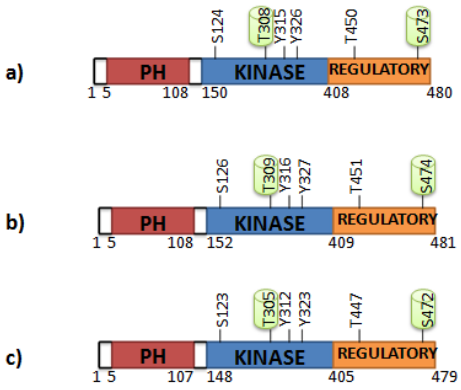

Figure 6: Figures a, b and c represent the domain structures of Akt1, Akt2, Akt 3 respectively (312).

These isoforms are produced by unique genes and with similar structures composed of three functional domains: a N-terminal pleckstrin homology (PH) domain, a central kinase domain, and a C-terminal regulatory domain including the hydrophobic motif (HM) phosphorylation site [FxxF(S/T)Y] (155). This pattern is preserved among various species including fly, worm, mouse, man. There is a high degree of conservation of central kinase domain and the HM among cAMP-dependent, cGMP-dependent and protein kinase C (ACG) members, such as protein kinase C, serum- and glucocorticoid-induced kinase, ribosomal p90 S6 kinase, and p70 S6 kinase, response regulators induced by insulin or other growth factors (164).

Akt kinase could interact with p70 S6 kinase to connect the biochemical and mechanical events in muscle contraction with downstream biological responses (205).

The PH domain is needed for the interaction of PIP3 preceeding the short glycine-rich region linked to the catalytic domain (206). The docking of PIP3 to the PH domain serves as the rate- limiting step in Akt activation, leading to the transport of Akt to the plasma membrane, in order to phosphorylate Akt at two vital residues for its complete activation (208). These residues are threonine (Thr-308 in Akt1) in the activation loop within the catalytic domain and serine (Ser-473 in Akt1) within the HM in the carboxy-terminal noncatalytic region (209).

It is interesting to note that the PH domain that is necessary for the association with lipids such as PtdIns (3.4.5)P3, but is absent in S. cerevisiae orthologue (Sch9) (177). Crystallographic analyses of Akt without the presence of the PH domain have indicated that a disordered αC helix in the N-terminal lobe of the catalytic domain affects Akt activation, irrespective of Thr-308 phosphorylation (178). However, the association of the HM with the N-terminal lobe is due to Ser-473 that causes a disordered-to-ordered transition of the αC helix (214). This transition produces the active kinase conformation (222).

Upon its stimulation, different sets of proteins can be targeted for phosphorylation by Akt including GSK3, Fox01, Fox03a, Fox04, and TSC2. Akt phosphorylation at Thr-308 and Ser-473 could be inhibited by TRB3 (179), which associates with the catalytic domain of Akt or carboxy-terminal mediated modulator protein (CTMP) (180), which interacts with the carboxy-terminal tail of Akt. It has been observed that the PH domain leucin-rich repeat protein phosphatase (PHLPP) dephosphorylates Ser-473 and inactivates Akt (181).

2.7.3 Functional properties of Akt

The serine/ thronine kinase Akt, is a downstream effector of PI3K (165). Extracellular signals such as growth factors, cytokines, hormones and neurotransmitters can cause Akt stimulation to form phosphatidylinositol 3' phosphate (PIP3) through the activation of PI3-Kinase (166). Tyrosine phosphorylation on p85α and p110 by insulin induces PI3-kinase activation (166). Specific stimuli like hyperosmolarity (167), growth hormone (168), heat shock (169), and reagents that elevate cAMP (170) lead to increased Akt activity through the PI3-kinase mechanism.

Akt activity is downregulated by the phospholipid phosphatases that dephosphorylate PIP3. The major phospholipid phosphatase is the tumor suppressor Phosphatase and Tensin homolog (PTEN), which dephosphorylates the 3' phosphate of PIP3 and disrupt the activity of PI3K (171). Wortmannin, a PI3-kinase inhibitor, blocks Akt activation and also its translocation (172). Studies have also observed that the insulin- or growth factor induced glucose transport is fully prevented on the negative regulation of p85α (171). Insulin- stimulated phosphorylation of Akt was affected in the skeletal muscle of insulin-resistant Goto-Kakizaki rats and in muscle biopsies from Type 2 diabetic patients. (172). This insulin resistance can be reversed by pholorizin administration that controls the high blood glucose levels in experimental diabetes by reducing the tubular reabsorption in glucose. Glucosamine, a product of the hexosamine pathway also inhibits the activation of Akt in endothelial cells (301).

In diabetic tissues, the other cause that can affect Akt activation is oxidative stress (301). A continuous inactivation of Akt by osmotic shock could be due to the activation of phosphatase activity without causing severe implications on the insulin receptor substrate, IRS1 tyrosine phosphorylation, or the activation of the PI 3-kinase (174). There is a 90% decrease in Akt phosphorylation due to the excessive H_2O_2 produced in adipocytes (175. 301). Tumor necrosis

factor-α (TNF-α), an inflammatory cytokine acts on the Akt levels by stimulating ceraminde, natural agonist of Akt phosphorylation (176).

The control of cellular metabolism regulated by insulin or IGF1 and other growth factors is the consistent function of Akt. Akt is involved in energy metabolism and is used in the apoptotic cascade in mammalian cells. Akt does not seem to mediate apoptosis in nematodes and flies (182). Akt plays a part in upregulating glucose uptake, glycolysis, and the association of mitochondrial hexokinases with the mitochondria (183).

In vitro studies suggest that Akt regulates insulin-stimulated glucose transport and dock to the GLUT4-containing vesicles (184). Akt overexpression causes elevated GLUT4 translocation and glucose uptake in muscles cells and adipocytes (185). Akt is an important mediator of insulin-induced GLUT4 exocytosis, while insulin-mediated GLUT4 endocytosis does not depend on Akt activation (186). Akt expression in adipocytes and muscle cells causes the uptake of 2-dehydoxy-D-glucose uptake, even without insulin-stimulation (187). Upon insulin treatment, adipocytes from obese patients with type II diabetes indicate a decrease in insulin-sensitivity and reduction in total phosphorylation of serine residues and Akt activation (188). In contrast to this, there was a 66 to 55% reduction in GLUT4 translocation to the plasma membrane in response to insulin with a microinjection of Akt substrate or anti-Akt antibody into the cells (189).

The transgenic expression of Akt increases the size of the islets in the pancreas due to hypertrophy and hyperplasia of islets (190). Thus, as Akt stimulation influences the islet β cell mass by changing the cell size and cell number, and has an effect on insulin secretion, Akt could be exploited to give rise to larger islet β cells in therapy (191). Akt injection into vascular endothelial cells increases the rate of angiogenesis (192), offers protection against oxidant-

induced injury when administered into the lung (196), and avoids remodeling and retrieves the efficiency of infracted hearts when introduced to stem cells (194). Overexpression of Akt in the heart leads to a reduction in the AMP- activated protein kinase activity and increased $p70^{S6K}$ phosphorylation (195).

The metabolism of lipids is regulated by Akt in the mammary gland (61). Studies from Alzheimer's disease (AD) brain samples have indicated that Aβ interferes with the activation reactions by affecting the association between phosphoinositide-dependent kinase (PDK) and Akt (195). By reversing the blockage of Akt binding with PDK pharmacologically, the insulin action in AD brain could be improved to a great extend (198).

Activated in many human cancers, the hyperactivation of Akt takes place through a variety of mechanisms. Mainly, elevated Akt kinase activity has been recorded in ~40% of breast, ovarian cancers (199), with a greater chance of occurrence in prostrate, glioblastoma, and melanoma, by inactivating the mutations or deletions of the tumor suppressor PTEN (200). Akt stimulation might lead to adjustment of cancer cells to their hypotoxic environment. In animal models, high levels of insulin in the blood elevate the Akt phosphorylation in breast cancer xenografts (201). This cause has been attributed to higher rates of tumor growth *ex vivo*, with chronic lymphoid leukemia (CLL) lymphocytes among humans who overexpress the insulin receptor (202). Some pro-apoptotic mediators like the transcription factor fork-head (FOXO), the tau kinase GSK-3β, and the Bcl2 antagonist Bcl-2-associated death promoter (BAD) proteins, are downregulated by Akt (61, 205, 223). It is now also established that Akt increases cell proliferation as well as cell growth in addition to its anti-apoptotic properties (191).

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Sequestosome 1 /p62 and TRAF6 serve as a bridge to connect IRS-1 with Akt in insulin signaling

3.1: Introduction

Insulin resistance is the key factor leading to the etiology of type 2 diabetes (8). Aberrations in insulin signaling pathway might result in obesity and type 2 diabetes (1). Phosphorylation of the insulin receptor (IR) occurs upon insulin stimulation (2). The insulin receptor substrate (IRS) family proteins (IRS-1 to IRS-4) are further phosphorylated by the activated IR. Most of the research highlights the functions of the insulin receptor substrate-1 (IRS-1) owing to its importance in the insulin-signaling pathway. The N-terminus pleckstrin homology domain of IRS-1 couples it to the insulin receptor (3, 4). The phosphotyrosine binding domain associates with the insulin-like growth factor receptors (4, 5). The phosphotyrosine (YXXM) motifs present in the C-terminal domain are phosphorylated by the insulin receptor and are the binding sites for the Src homology-2 (SH_2) domain-containing proteins (41). Enzymes including phosphatidylinositol (PI) 3-kinase, Grb-2, SHP-2, Fyn, and Nck interact with IRS-1 stimulating the downstream signaling cascade pathway of insulin (6, 7). The phosphatidylinositol 3-kinase (PI3K) results in the formation of phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$). This leads to the movement of Akt to the plasma membrane (PM) where the mammalian target of rapamycin (mTOR) complex 2 (mTORC2) and the phosphoinositide-dependent kinase 1 phosphorylates it at Thr-308 and Ser-473 (9, 10). Insulin receptor substrate-1 (IRS-1) connects the insulin receptor (IR) to the PI3K and the MAPK insulin dependent pathways (31). Glycogen synthase kinase 3 (GSK3), the Rab GTPase-activating

protein (RabGAP), AS160, also known TBC1D4, proline-rich Akt substrate of 40 kDa (PRAS40), and RhebGAP tuberous sclerosis protein 2 (TSC2) are phosphorylated by the activated Akt. Akt activation translocates GLUT4 from cytosol to membrane and enhances glucose uptake (11, 12).

Sequestosome 1/p62 plays a vital role as a regulating protein and is involved in receptor-mediated signal transduction. p62 is a 62-kDa protein that acts as a scaffolding protein due to its association with the atypical PKCs in different signaling pathways (15). Initially p62 was observed as a phosphotyrosine-independent ligand of the SH_2 domain of p56 lck (16). The domain structure of p62 is responsible for its ability to associate with target proteins (17, 18). It includes an ubiquitin-like domain (UBL) at its N-terminus (19), and within this UBL domain, there is a SH_2 binding domain and an acidic interaction domain that associates with the atypical PKC (15, 20). The ZZ finger, a docking site for the RING finger protein tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), two peptide sequences rich in proline, glutamic acid, serine, and threonine (PEST sequences), an LC3-association region (21) and the C-terminus includes the ubiquitin-associated (UBA) domain (19). p62 gene knockout in the gene causes obesity progressing to insulin resistance and type 2 diabetes (22, 23). The disruption of p62 gene in mice results in an Alzheimer's phenotype (13).

Recently in our lab, we have shown that SH_2 domain of p62 interacts with YXXM motif of IRS-1 (8). p62 is also shown to participate in the insulin signaling pathway (8). TRAF6, ubiquitin ligase is known to interact with p62 through the TRAF6 interacting domain (27). TRAF6 serves as an ubiquitin ligase for Akt ubiquitination and phosphorylation (30). p62 serves as a scaffolding protein to induce polyubiquitination and oligomerization of TRAF6 therby

increasing the ligase activity of TRAF6 (32). In this study, we report that p62/TRAF6 complex serves as a bridge to connect IRS-1 to Akt in insulin signaling. p62 and TRAF6 plays a novel role in the activation of Akt by insulin.

3.2: Materials and Methods

Antibodies and Reagents

Anti-IRS1 was purchased from Millipore, Temecula, CA and anti-p62, anti-V5, anti-HA, anti-Myc were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Phospho-Akt (Thr- 308 and Ser-473) and total Akt antibody were purchased from Cell Signaling Technology (Danvers, MA), TRAF6 antibody from Abcam (Cambridge, MA). Anti-rabbit IgG and anti-mouse IgG-HRP linked secondary antibody were obtained from GE Healthcare UK Ltd., and enhanced chemiluminescence (ECL) was from Thermo Scientific. Protein A-Sepharose beads and all other reagents were obtained from Sigma-Aldrich.

Cell Culture

Parental L6 cells were maintained in DMEM medium (Invitrogen), and Chinese Hamster Ovary (CHO) cells overexpressing the human IR (CHO/IR) were grown in Ham's F-12 nutrient mixture (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Wild type and TRAF6 knockout Mouse Embryonic Fibroblast cells (MEF cells) were obtained from Dr. Inoue, University of Tokyo while p62 knockout MEF cells were obtained from Dr. Ishii, University of Tsukuba. L6 myoblasts were cultured in DMEM medium with 2% fetal bovine serum, penicillin/streptomycin for 7 days and were changed on a daily basis in order to stimulate the differentiation into myotubes. Cationic lipid method with Lipofectamine^{TM} 2000 transfection reagent (obtained from Invitrogen) was used to transfect

CHO/IR cells. The cells were starved in a serum-free culture medium for 4 h at 37 °C before cell lysis.

Immunoprecipitation and Western Blotting Analysis

Cells were treated with or without insulin (100 nM) for 15 min at 37 °C. Triton lysis buffer (50 mM HEPES (pH 7.6), 150 mM NaCl, 20 mM sodium pyrophosphate, 10 mM NaF, 20 mM 13-glycerophosphate, 1% Triton, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml leupeptin and aprotinin) was used to lyse the cells. Bradford procedure (Bio-Rad) was used to estimate the protein with bovine serum albumin (Sigma-Aldrich) as a standard. The cell lysates (1 mg) were incubated with 4 µg of the primary antibody. The immunoprecipitates were collected overnight with protein A-Sepharose beads at 4 °C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to the samples and boiled. The proteins were resolved on 10% SDS-PAGE, transferred to PVDF membrane (Millipore) and western blotted using suitable antibodies.

3.4: Results

3.4.1: Insulin elicits the interaction of TRAF6 with IRS-1 and Akt

In our laboratory, we recently found that IRS-1 interacts with p62 (8). Because p62 can interact with TRAF6 (27), we sought to identify whether TRAF6 can associate with IRS-1 and Akt upon insulin stimulation. L6 myotubes were treated with or without insulin (100 nM) for 15 min at 37 °C after serum-starvation for 4 hours. Triton lysis buffer was used to lyse the cells and immunoprecipitation was performed using anti-IRS-1 antibody. The immunoprecipitates were western blotted with anti-IRS-1, anti-Akt and anti-TRAF6 antibody. Results indicated that TRAF6 interacts with IRS-1 and Akt upon insulin stimulation (Fig. 7*A*). In the absence of

insulin, there was no association between TRAF6, IRS-1 and Akt. The first two lanes of each blot was loaded with the crude lysates.

To further confirm these interactions, CHO/IR cells were transfected with V5-IRS-1 and Flag-TRAF6. The cells were stimulated with or without insulin for 15 min as shown in Fig. 7*B*. V5 was immunoprecipitated after cell lysis, following which western blotting was performed with V5 and Oct-A antibody. The whole cell lysates were also western blotted with myc, V5 and Oct-A to confirm the transfection of the respective constructs. These results suggest that TRAF6 interacts with IRS-1 and Akt upon insulin stimulation (Fig. 7B). Further, CHO/IR cells were transfected with myc-Akt and/or Flag-TRAF6, and treated with insulin as shown in figure 7C. The cells were lysed and immunoprecipitated with myc antibody and western blotted with myc and Oct-A. Oct-A antibody recognizes the Flag epitope. Results showed that Akt interacts with TRAF6 upon insulin stimulation. The cells without insulin treatment did not demonstrate any detectable Akt-TRAF6 complex formation (Fig. 7C).

3.4.2: TRAF6 is essential for the interaction of p62 with Akt

The previous results supported the evidence of an interaction between TRAF6, IRS-1 and Akt. Further, we intended to identify the importance of TRAF6 for the interaction of p62 and Akt to occur. L6 myotubes were serum-starved for 4 hours, the cells were treated with or without insulin (100 nM) for 15 min at 37 °C. p62 was immunoprecipitated and western blotting was performed using anti-IRS-1, anti-TRAF6, anti-Akt and anti-p62 antibodies. The input samples were loaded as crude lysates to show the uniformity of endogenous IRS-1, TRAF6, p62 and Akt. Results show that p62 interacts with IRS-1, TRAF6, Akt upon insulin stimulation. In the absence of insulin, there was no interaction of IRS-1, TRAF6, Akt with p62 (Fig. 8*A*). To further confirm that p62 interacts with Akt, CHO/IR cells were transfected with myc-Akt and

HA-p62 upon insulin stimulation; followed by the immunoprecipitation of lysates with myc antibody and western blotted with myc and HA which are tags to detect the Akt and p62 constructs. Results from this transfection indicated that p62 interacts with Akt upon insulin stimulation (Fig. 8B). Cells lysates were also western blotted with HA and myc antibody to check the expression of Akt and p62 constructs. To verify whether TRAF6 is required for p62 to interact with Akt, we used wild type and TRAF6 knockout MEF cells. Wild type and TRAF6 knockout MEFs were stimulated with or without insulin (100nm) for 15min at 37°C followed by immunoprecipitation (IP) with Akt antibody and western blotted with p62, TRAF6 and Akt. In wild type MEFs, p62 interacts with Akt on insulin stimulation. However in TRAF6 knockouts, the interaction between p62 and Akt is impaired (Fig 8C). This explains that TRAF6 connects p62 with Akt. As the control, the inputs were used to confirm the absence of TRAF6 in TRAF6 knockout MEF lysates. These data suggest that the interaction of p62 with Akt is dependent upon TRAF6.

3.4.3: TRAF6 interacts with IRS-1 through p62

We then sought to examine if TRAF6 was crucial to connect Akt to p62 and IRS-1. CHO cells that overexpressed the insulin receptor were transfected with V5-IRS-1, Flag-TRAF6, myc-p62 and ASp62. The transfected cells were stimulated with insulin (100 nM) for 15 min. The interaction of IRS-1 with TRAF6 was determined by immunoprecipitation using V5 antibody and western blotting with Oct A antibody. The results in figure 3A indicate that the interaction of TRAF6 with IRS-1 increases upon the overexpression of p62. The reduction of p62 expression due to the transfection of anti-sense p62 (ASp62) decreased the association of TRAF6 with IRS-1 significantly. Immunoblots of the input samples are the crude lysates from the transfected cells confirms the expression of the transfected constructs (Fig. 9A). This suggests

that the interaction of IRS-1 with TRAF6 is dependent upon p62. To further confirm this, CHO/IR cells were transfected with ASp62, and treated with or without insulin treatment. TRAF6 was immunoprecipitated, and western blotting was performed to detect IRS-1, p62, Akt and TRAF6. TRAF6 interacted with IRS-1, p62 and Akt on insulin stimulation (Fig. 9B). However, when p62 expression was reduced, TRAF6 interacted with Akt but not with IRS-1. In order to further support this evidence, wild type and p62 knockout MEFs were stimulated with or without insulin (100nm) for 15min at 37°C followed by immunoprecipitation (IP) with TRAF6 antibody and western blotted with IRS-1, p62, TRAF6 and Akt. In wild type MEFs, TRAF6 interacts with IRS-1 and Akt on insulin stimulation. However in p62 knockouts, the interaction between TRAF6 and IRS-1 is impaired (Fig. 9C). This result again explains that p62 is essential for a significant interaction to occur between IRS-1 and TRAF6. Further, TRAF6 interacted with Akt even in the p62 knockout MEFs (Fig. 9C). As the control, the inputs were used to confirm the absence of p62 in the p62 knockout MEF lysates. This clearly explains that the interaction of TRAF6 with IRS-1 is dependent upon p62 but not its interaction with Akt.

3.4.4: p62 and TRAF6 are essential for Akt activation

To examine whether p62 and TRAF6 serve as a bridge to connect IRS-1 with Akt, we used wild type, TRAF6 knockout and p62 knockout MEFs. These MEFs were stimulated with or without insulin (100 nM) for 15 min at 37°C followed by immunoprecipitation (IP) with IRS-1 antibody and immunoblotting with IRS-1, p62, TRAF6 and Akt (Fig. 10A). In wild type MEF, IRS-1 forms a complex with p62, TRAF6 and Akt only upon insulin stimulation. In p62 knockout, IRS-1 does not interact with TRAF6 and Akt whereas in TRAF6 knockout, IRS-1 interacts with p62 but not Akt. As control, the expression level of p62, TRAF6, Akt, IRS-1 was examined

employing equal protein concentrations of whole cell lysates (Fig. 10A). These results suggest that p62 and TRAF6 links IRS-1 to Akt.

To further examine whether p62 and TRAF6 are involved in Akt activation, wild type, p62 knockout and TRAF6 knockout MEF cells were treated with or without insulin and immunoblotted for phospho and total Akt. In the wild type MEF cells, the phosphorylation of Akt at Thr-308 and Ser-473 was enhanced upon insulin treatment. However, the knockdown of p62 and TRAF6 significantly decreased the phosphorylation of Akt in comparison with wild type MEFs (Fig. 10*B*). The immunoblot of the total Akt from the lysates served as a loading control. These results clearly suggest that p62 and TRAF6 are essential for Akt activation.

3.5: Discussion

After a meal, insulin is involved in regulating the glucose from the plasma. Upon insulin action, the PI 3-kinase gets activated and the insulin signaling cascade is initiated. Through phosphorylation, protein-protein interactions, and protein modifications, the insulin receptor substrate-1 (IRS-1) is involved in delivering the insulin signal (8). IRS-1-p62 complex formation occurs upon insulin stimulation and sequestosome 1/p62 has been identified to participate in the insulin signaling pathway (8).

In this study, we report that p62 can interact with TRAF6, which in turn can bind Akt upon insulin stimulation. p62 interacts with TRAF6 in response to insulin and may likely serve as a bridge between IRS-1 and Akt. p62 and TRAF6 are essential to activate Akt upon insulin stimulation. p62 is known to interact with TRAF6 through its TRAF6 interacting domain (27). Akt is found to be a substrate of TRAF6 (30). TRAF6 is necessary to Akt ubiquitination, phosphorylation and translocation to membrane (30). Akt is an essential kinase involved in

GLUT4 translocation and glucose uptake (14, 35). p62 serves as a scaffolding protein to enhance the ligase activity of TRAF6 (32). Thus, the E3 ubiquitin ligase activity of TRAF6 may be inhibited by the loss of p62. This may in turn decrease the Akt phosphorylation in p62 or TRAF6 knockout MEF cells as indicated in Fig. 10*B*. p62 and TRAF6 are essential for the phosphorylation of Akt at T308 and S473. The absence of even one among the two proteins affects the activation of Akt significantly due to the modification in the mode of interaction and in the downstream signaling cascade.

Thus, by combining these results indicating the interaction of these chief proteins involved in the insulin signaling pathway, p62 and TRAF6 has been found to link IRS-1 to Akt. These four vital proteins exist as a complex in the pathway. Deletions or modifications in the complex can thus affect the entire downstream signaling, indicating the major implications and the connection of these proteins to type 2 diabetes.

3.6: Figures

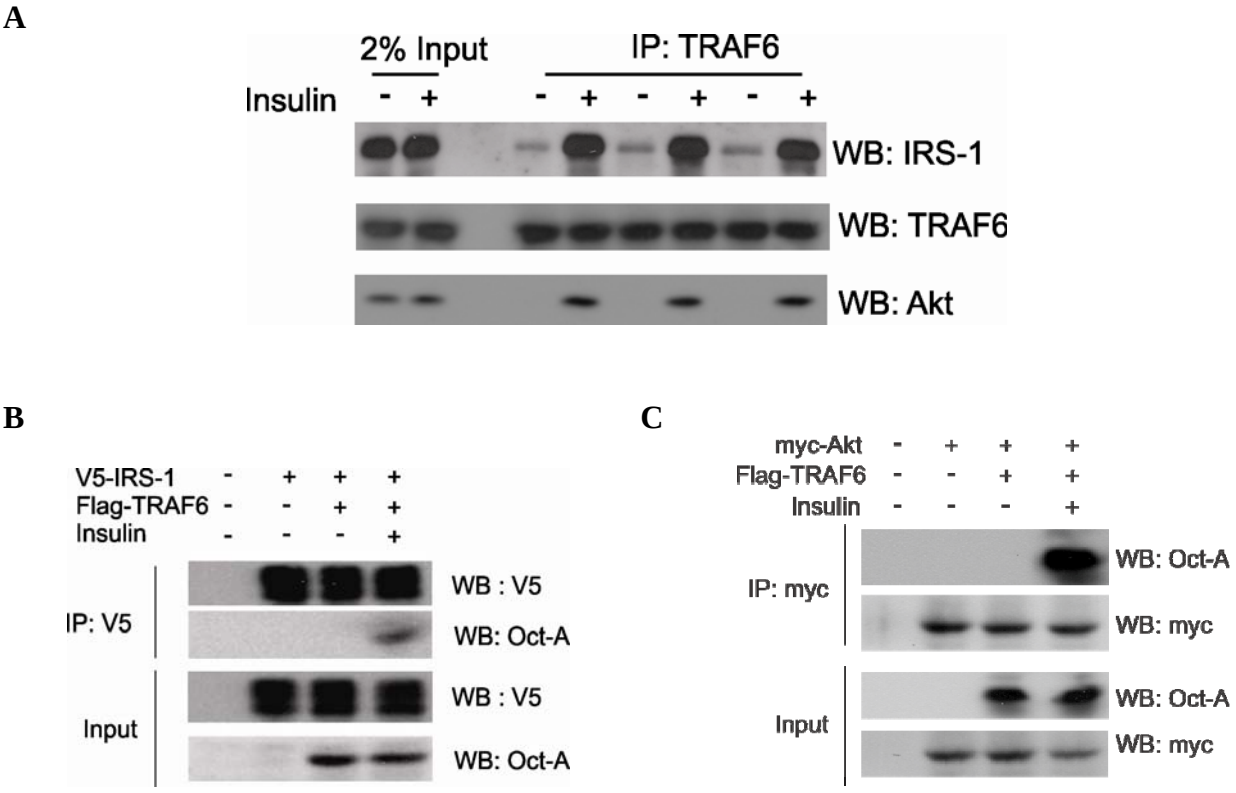
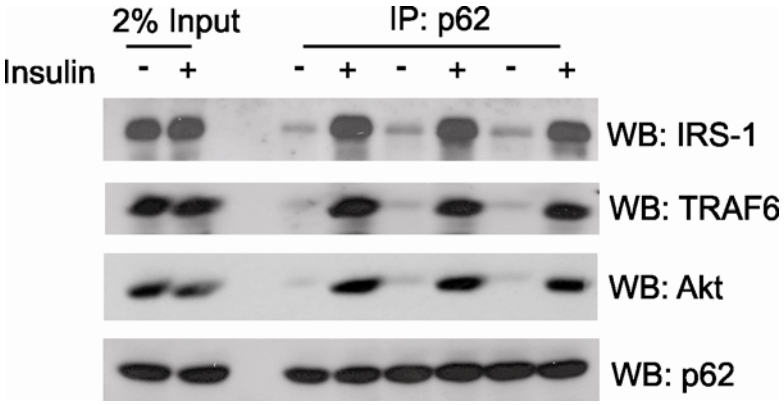


Figure 7: Interaction of TRAF6 with IRS-1 and Akt. A) L6 myotubes were serum-starved for 4 h and either left untreated or stimulated with insulin (100 nm) for 15 min at 37°C. One mg of lysate protein was immunoprecipitated (IP) with TRAF6 antibody and western blotted with antibodies IRS-1, Akt or TRAF6. The lanes labeled input indicate crude lysate. B) CHO/IR cells were transfected with V5-IRS-1 and Flag-TRAF6 followed by insulin (100 nM) stimulation for 15 min at 37°C. The lysates were immunoprecipitated with V5 antibody and immunoblotted with V5 and Oct-A to detect the tagged IRS-1 and TRAF6 constructs. Whole-cell lysates were separated by SDS-PAGE and immunoblotted (WB) with V5, Oct-A antibody. C) CHO/IR cells were transfected with myc-Akt and Flag-TRAF6. After 48 hours, the cells were treated with or without insulin (100 nM) for 15 min at 37°C. The lysates were immunoprecipitated with myc antibody and immunoblotted with myc and Oct-A to detect the tagged Akt and TRAF6 constructs. Whole-cell lysates were separated by SDS-PAGE and immunoblotted (WB) with myc and Oct-A antibody.

A



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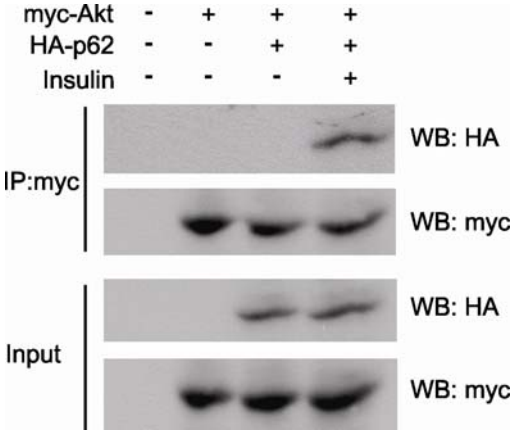

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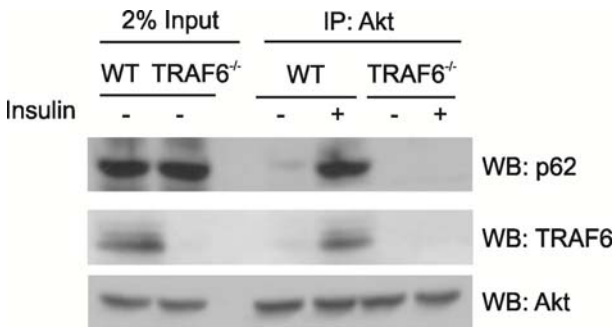
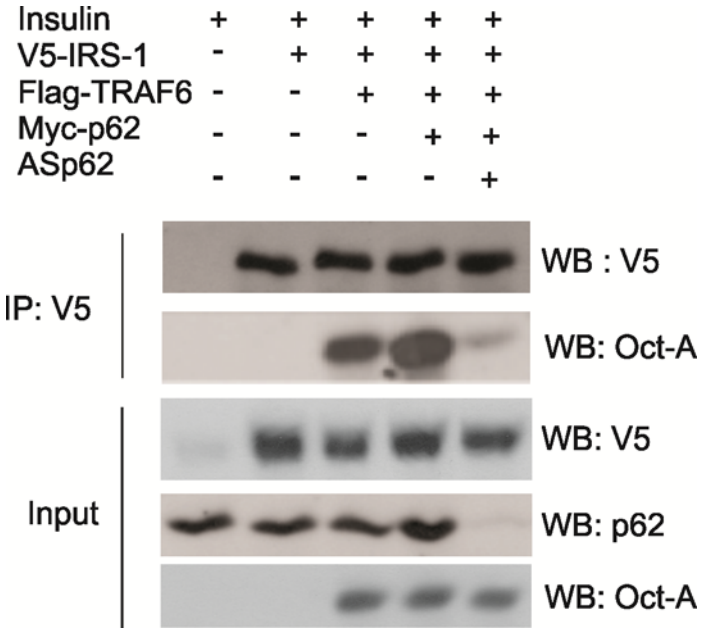


Figure 8: Interaction of p62 with AKT is through TRAF6. A) L6 myotubes were treated with insulin (100 nM) for 15 min at 37°C or not followed by immunoprecipitation (IP) with p62 antibody and immunoblotting with IRS-1, TRAF6 and Akt. B) CHO/IR cells were transfected with myc-Akt and/or HA-p62. The cells were treated with or without insulin (100 nM) for 15 min at 37°C. The lysates were immunoprecipitated with myc antibody and immunoblotted with HA and myc to detect the interaction between tagged p62 and Akt constructs. Whole-cell lysates were separated by SDS-PAGE and immunoblotted (WB) with HA and myc antibody. C) Wild type and TRAF6 knockout Mouse Embryonic Fibroblast cells were stimulated with or without insulin (100 nM) for 15 min at 37°C. The MEFs were lysed and immunoprecipitated with Akt antibody and immunoblotted with p62, TRAF6 and Akt. As the control, the expression of the proteins in the MEFs was determined in the input.

A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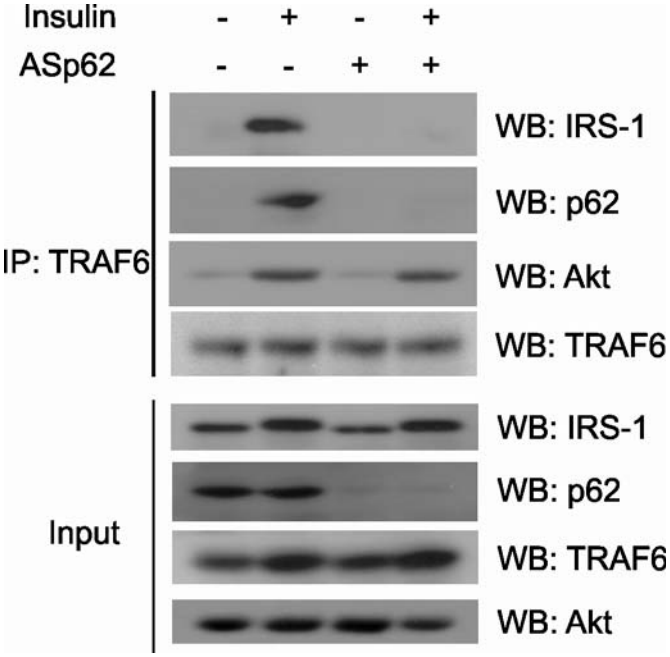
V5-IRS-1 - + + + +
Flag-TRAF6 - - + + +
Myc-p62 - - - + +
ASp62 - - - - +
IP: V5
WB : V5
WB: Oct-A
Input
WB: V5
WB: p62
WB: Oct-A

B


Insulin - + - +
ASp62 - - + +
IP: TRAF6
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WB: Akt
WB: TRAF6
Input
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WB: TRAF6
WB: Akt

C

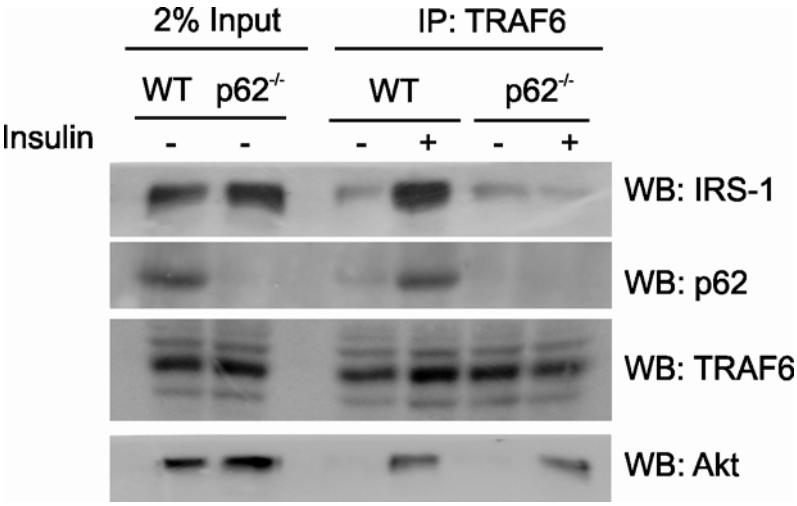
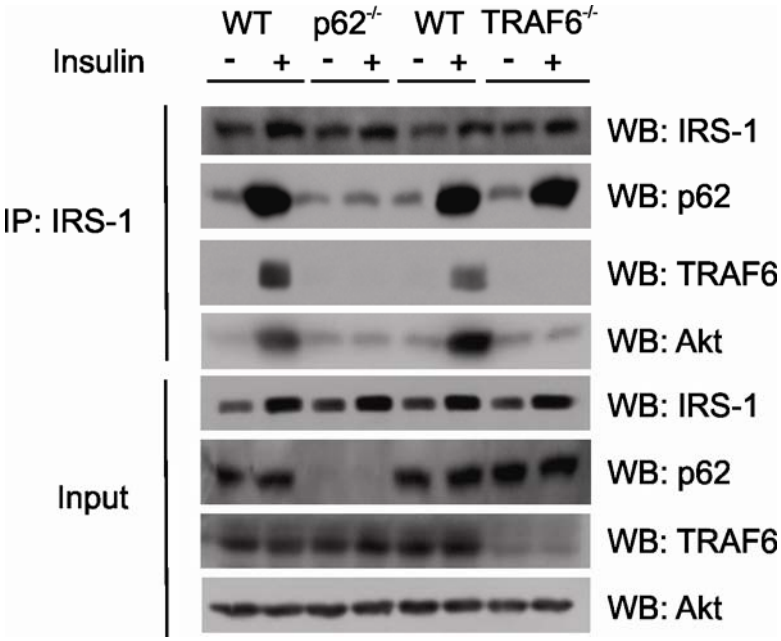


Figure 9: TRAF6 links Akt to p62 and IRS-1. A) CHO/IR cells were transfected with V5-IRS-1, Flag-TRAF6, myc-p62 and were treated with insulin (100 nM) for 15 min. Cells were lysed and immunoprecipitated with V5 antibody and immunoblotted with V5 and Oct A tags for IRS-1 and TRAF6 constructs. Whole-cell lysates were lysed and immunoblotted (WB) with V5, p62, Oct A antibody to verify the expression of the constructs. B) CHO/IR cells were transfected with antisense p62 (ASp62) and treated with insulin (100 nM) for 15 min or not. The lysates were immunoprecipitated with TRAF6 antibody and immunoblotted with IRS-1, p62, TRAF6 and Akt. Whole-cell lysates were separated by SDS-PAGE and immunoblotted (WB) with all these antibodies. C) Wild type and p62 knockout Mouse Embryonic Fibroblast cells were stimulated with or without insulin (100 nM) for 15 min at 37°C. The MEFs were lysed and immunoprecipitated with TRAF6 antibody and immunoblotted with IRS-1, p62, TRAF6 and Akt. As the control, the expression of the proteins in the MEFs were determined in the input.

A



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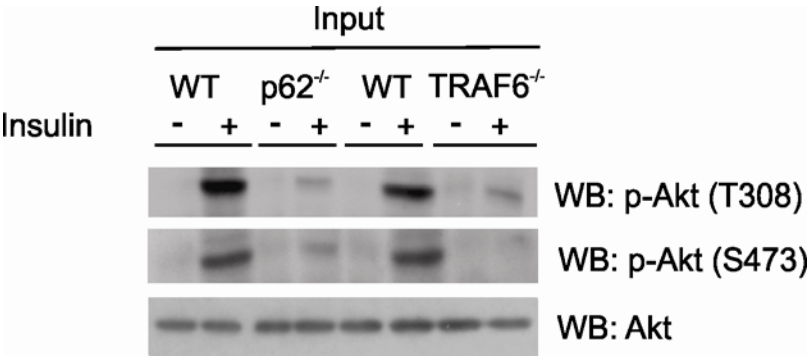

Figure 10: p62 and TRAF6 are necessary to activate Akt. A) Wild type, TRAF6 knockout and p62 knockout MEFs were stimulated with or without insulin (100 nM) for 15 min at 37°C. IRS-1 was immunoprecipitated and immunoblotted with IRS-1, p62, TRAF6 and Akt. As the control, the expression of the proteins in the lysate was determined. B) Wild type, TRAF6 knockout and p62 knockout Mouse Embryonic Fibroblast cells were stimulated with or without insulin (100 nM) for 15 min at 37°C followed by immunoblotting with p-Akt (T308), (S473) and Akt antibody.

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