

Sequestosome 1/p62 and TRAF6 are necessary for Akt ubiquitination, translocation and activation

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## Abstract

Defects in protein-protein interactions of the insulin signaling pathway result in insulin resistance that precedes type 2 diabetes. The protein Akt is the primary modulator of cellular glucose uptake through the GLUT4 transporter. On insulin stimulation, Akt is reported to undergo ubiquitination by the TRAF6 ligase before recruitment to the membrane. We have previously shown in our lab that p62 and TRAF6 serve as a bridge to connect IRS-1 with Akt. TRAF6 is known to form a complex with p62, modulating activation and increasing E3 ubiquitin ligase activity. Investigations in L6 myotubes and TRAF6<sup>-/-</sup> and p62<sup>-/-</sup> Mouse Embryonic Fibroblasts (MEF) indicate p62 and TRAF6 serve in Akt ubiquitination on insulin stimulation. Further study in TRAF6<sup>-/-</sup> and p62<sup>-/-</sup> MEF cells confirmed interactions with p62 and TRAF6 are necessary for Akt translocation and activation on insulin stimulation. Wild-type MEF cells stimulated with insulin exhibited Akt membrane recruitment and activation, whereas insulin stimulated TRAF6<sup>-/-</sup> and p62<sup>-/-</sup> MEF cells exhibited impaired membrane recruitment and activation of Akt. Therefore, the TRAF6/p62 complex is necessary for Akt ubiquitination, translocation to the plasma membrane, and activation.

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## **CHAPTER 1: INTRODUCTION**

Chronic, non-communicable diseases are dramatically increasing on a global scale.<sup>1,2</sup> Substantial economic growth and revolutionary food production practices have generated world-wide nutritional transitions.<sup>3</sup> Global nutritional goods and services are increasingly based on Westernized practices resulting in increased processed food intakes and reduced intake of traditional meals.<sup>4</sup> Westernization and the expansion of industrialization have substantially facilitated escalating world-wide obesity and diabetes prevalence rates.<sup>5</sup> According to the International Diabetes Federation, 382 million people are living with diabetes and prevalence rates are projected to increase to 592 million by the year 2035.<sup>6</sup>

The rising prevalence of diabetes and debilitating complications of the disease have sparked international attention.<sup>2,7</sup> Diabetes was estimated to cause 6.8% of global deaths in individuals 20-79 years of age in 2010.<sup>8</sup> Diabetes doubles the risk of death of an individual in any age group compared with an aged matched individual without diabetes.<sup>9</sup> Urgency is essential in diagnosed patients, as prolonged, un-controlled diabetes may result in retinopathy, lower limb amputation, renal failure, and cardiovascular disease.<sup>10,11</sup> These diabetes complications cause disability, impair quality of life, and are life-threatening.<sup>11</sup> The detrimental complications of diabetes and alarming prevalence rates fortify the need to advance understanding of diabetes to mitigate this complex disease.

Decades ago, diabetes was associated with affluence, but diabetes is now a burden on low-income populations.<sup>6</sup> An estimated 80% of diabetes patients live in low to middle income countries and 84% are estimated to be undiagnosed.<sup>6</sup> Arbitrary symptoms and inadequate healthcare systems make diabetes awareness campaigns essential to combating world-wide prevalence.<sup>1,4</sup>

Type 2 diabetes mellitus (T2DM) is the seventh leading cause of death in the United States.<sup>12</sup> According to the American Diabetes Association, 8.3% of the U.S. population has diabetes and 35% of Americans 20 or older have pre-diabetes.<sup>13</sup> Moderate estimates of future diabetes prevalence indicates an increase to 25-28% by 2050.<sup>14</sup> The medical cost of diabetes in the U.S. reached an estimated \$245 billion in 2012 according to the American Diabetes Association.<sup>15</sup> Data taken from the National Health Interview Survey found the prevalence rate of Hispanic and non-Hispanic black ethnicities is 12% compared with the prevalence rate of 7.4% among non-Hispanic whites.<sup>16</sup> Diabetes is the leading cause of blindness, kidney failure, neuropathy, and non-traumatic lower-limb amputation in the U.S.<sup>13</sup>

The state of Alabama was ranked as the 5<sup>th</sup> state with the highest diabetes prevalence in 2010 with a 11.1% prevalence rate.<sup>17</sup> The Alabama Health Disparities Report in 2010 reported challenging healthcare disparities impede reducing diabetes prevalence rates in the state of Alabama.<sup>17</sup> Rural areas of Alabama are reported to lack local healthcare facilities and an adequate amount health care professionals confounding diabetes interventions.<sup>17</sup>

Type 1 diabetes mellitus (T1DM) makes up 5-10% of diabetes cases in the United States.<sup>12</sup> T1DM is characterized as a lack of circulating insulin due to destruction of insulin

secreting pancreatic  $\beta$ -cells.<sup>18,19</sup> Untreated patients experience hyperglycemia ketoacidosis, ketouria, polydipsia, polyphagia, and wasting.<sup>10,20</sup> The only treatment of T1DM is exogenous insulin therapy.<sup>20,21</sup>

T2DM is a multi-factorial disease making up 90-95% of diabetes cases in the United States.<sup>12</sup> Insulin resistance and dysfunctional  $\beta$ -cells are defining characteristics of T2DM.<sup>22</sup> High blood glucose (hyperglycemia) is a hallmark of T2DM.<sup>23</sup> Hyperglycemia produces symptoms including: polyuria, polydipsia, polyphagia, fatigue, blurred vision, slow healing of sores, and frequent infections.<sup>24</sup> Obesity and insulin resistance are major risk factors of T2DM.<sup>25,26</sup> Diet and exercise can prevent and reverse T2DM and several medical therapies have become available to mitigate the disease.<sup>27-29</sup>

Insulin serves as a growth hormone which signals glucose uptake in skeletal, adipose, and hepatic tissue.<sup>30</sup> The action of insulin is impaired in T2DM patients causing metabolic abnormalities.<sup>31,32</sup> Insulin impairment, known as insulin resistance, has been proposed as the underlying cause of obesity and cardiovascular disease.<sup>33</sup> The cause of insulin resistance is not clearly understood, although evidence suggests some individuals may have defects in the insulin signaling pathway.<sup>32,34</sup> Insulin docks to insulin receptors on the plasma membrane inducing a conformational change which elicits an intra-cellular cascade.<sup>35</sup> Insulin acts on cellular insulin receptors to stimulate the glucose transporter GLUT4 to the cellular membrane.<sup>36</sup> GLUT4 transports glucose into skeletal, adipose, and hepatic tissue for cellular energy homeostasis.<sup>37</sup> There remains several gaps in understanding molecular interactions in the insulin signaling pathway.<sup>38</sup> Understanding the molecular mechanisms of GLUT4 activation and translocation to the cell membrane may lead to new therapeutic targets in diabetes research.

Akt/Protein Kinase B is a major cellular modulator protein in the insulin signaling pathway.<sup>39</sup> Akt is crucial for cell survival, and Akt serves in protein transcription, nutrient metabolism, cell proliferation, and anti-apoptotic pathways.<sup>39,40</sup> Akt modulates cell metabolism in the highly conserved PI3K/Akt/mTOR pathway through kinase activity.<sup>40</sup> Activation of Akt upon insulin stimulation is necessary for GLUT4 translocation to the cellular membrane.<sup>32</sup> The importance of Akt in diabetes pathology is exemplified in Akt2 knockout (KO) mice.<sup>41</sup> Akt2 KO mice exhibit severe glucose intolerance preceding  $\beta$ -cell dysfunction and diabetic complications.<sup>41</sup>

Sequestosome 1/p62 (p62) serves as a modulator, adaptor, and scaffolding protein.<sup>42,43</sup> p62 is comprised of a variety of protein domains allowing p62 to function in multiple signaling cascades.<sup>43</sup> p62 has been identified as a modulator protein in diseases such as: Alzheimer's Disease, Paget's Disease, obesity, breast cancer, and Parkinson's Disease.<sup>44</sup> Recent evidence from our lab indicates p62 mediates signal transduction from IRS-1 to Akt in the insulin signaling cascade.<sup>45</sup> p62 binds with TRAF6 forming the TRAF6/p62 complex facilitating substrate K63 ubiquitination.<sup>46,47</sup> p62 also binds with K48 ubiquitinated substrates in the ubiquitin/proteasome pathway and shuttles the tagged proteins to proteasomes for degradation.<sup>48</sup>

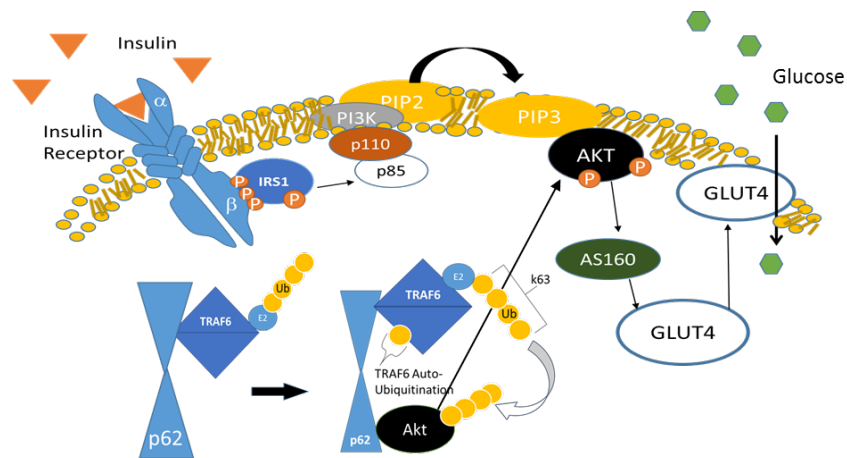
TNF- $\alpha$  receptor associated factor 6 (TRAF6) is an E3 ubiquitin ligase involved in mediating signal transduction in several cellular transduction pathways.<sup>49-51</sup> TRAF6 modulates pro-inflammatory proteins in the TNF receptor super family and interleukin-1 receptor superfamily.<sup>47,52</sup> TRAF6 is also involved in immunity through B lymphocyte modulation of CD-40 and bone formation through modulation of AP-1 protein activation after RANK-L stimulation.<sup>53,54</sup>

Ubiquitin is a highly conserved cellular signal that forms polymer chains that attach to target proteins.<sup>55</sup> Non-covalent binding of ubiquitin monomer or polymer chains serves in cell signaling, trafficking, and activation.<sup>56,57</sup> Evidence indicates the lysine linkage of polymer ubiquitin chains signal the fate of tagged proteins.<sup>48</sup> Research has indicated Akt must undergo ubiquitination for activation.<sup>58</sup>

The goal of this study was to determine whether the p62/TRAF6 complex is necessary for ubiquitination of Akt which in turn leads to Akt translocation and activation. Results of this research will enhance understanding of the insulin signaling cascade and may be implemented in future therapeutic diabetes research.

## Objective and Hypothesis

Our first objective was to determine if Akt is polyubiquitinated on insulin stimulation. Second, we analyzed if Akt is a substrate of TRAF6/p62 complex. Third, we investigated TRAF6 ubiquitination on insulin stimulation. Fourth, we investigated whether the TRAF6/p62 complex is necessary for Akt translocation to the plasma membrane and its phosphorylation. We hypothesized that the TRAF6/p62 complex is necessary for Akt ubiquitination, translocation, and activation.



**Figure 1: Schematic representation of hypothesized insulin signaling.** Upon insulin stimulation TRAF6 interacts with p62 and forms a complex. This p62/TRAF6 complex drives ubiquitination of Akt, which leads to its translocation and phosphorylation.

## **CHAPTER 2: REVIEW OF LITERATURE**

### **2.1 Diabetes Mellitus**

#### **2.1.1 Epidemiology**

The term Diabetes Mellitus represents a spectrum of disorders involving  $\beta$ -cell dysfunction and insulin resistance.<sup>60</sup> The most common types of diabetes mellitus are known as type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM).<sup>60</sup> T1DM is characterized as a lack of insulin secreting  $\beta$ -cells due to  $\beta$ -cell destruction, and T2DM is characterized as a dysfunction in  $\beta$ -cell secretion and insulin resistance.<sup>60</sup> The International Diabetes Federation estimates diabetes affects 382 million individuals world-wide.<sup>6</sup> Diabetes is the 7<sup>th</sup> leading cause of death in the U.S and reached an estimated economic burden of \$245 billion in 2012.<sup>15,61</sup> Diabetes increases risk of cardiovascular disease, which is the leading cause of death in the United States.<sup>62,63</sup> Untreated diabetes may result in debilitating complications including: kidney failure, blindness, or amputation of the lower extremities.<sup>61,64</sup> Complications of diabetes are now a leading cause of morbidity and mortality world-wide.<sup>65</sup>

Increased wealth and infrastructure has stimulated a world-wide nutritional transition towards food abundance.<sup>3</sup> Rates of processed food and sugar consumption are rising, contributing to a global increase in non-communicable disease.<sup>5</sup> China and India have the largest populations with diabetes in the world.<sup>6</sup> Ninety-eight million people in China and sixty-

five million people in India were predicted to have diabetes in 2013.<sup>6</sup> The prevalence rate of diabetes within the Chinese population reached 9.6% in 2013; however, the country with the highest prevalence rate is in Micronesia where a predicted 37.3% of the population have diabetes.<sup>6</sup> The global incidence rate of diabetes is projected to rise from 366 million to 592 million by the year 2035.<sup>6</sup>

The United States has the third highest population rate of diabetes in the world and a prevalence rate of 8.3%.<sup>6</sup> The high prevalence of diabetes in the United States places a heavy economic burden on government programs and the United States economy.<sup>15</sup> Diabetes treatment accounts for 176 billion dollars in government sponsored medical costs.<sup>15</sup> Decreased patient productivity increases the economic burden of diabetes by an estimated 69 billion dollars.<sup>15</sup> Alabama was ranked as the 5<sup>th</sup> highest prevalence rate in U.S. in 2010.<sup>61</sup> The Centers for Disease Control (CDC) reported 11.1% of residents in the state of Alabama were diagnosed with diabetes in 2010 and 7% were diagnosed with pre-diabetes.<sup>61</sup>

### **2.1.2 Type 1 Diabetes**

T1DM is a multi-factorial disorder characterized by an absence of insulin secretion due to  $\beta$ -cell destruction.<sup>60</sup> T1DM makes up 5-10% of diabetes cases in the United States.<sup>60,61</sup> The majority of patients with T1DM are diagnosed before young adulthood.<sup>18</sup> The cause of T1DM is largely unclear, but evidence indicates genetic polymorphisms account for a portion of diabetes cases.<sup>18,60</sup> Development of T1DM slowly progresses over time and is facilitated by an autoimmune response against pancreatic  $\beta$ -cells.<sup>18</sup> Autoantibodies that lead to autoimmune  $\beta$ -cell destruction can be detected in T1DM patients years before symptoms present themselves.<sup>66</sup>



Environmental factors trigger leukocytes infiltration of pancreatic islets and destruction of  $\beta$ -cells leading to diminished insulin production.<sup>18,67</sup> Several environmental factors including diet and viral infections are associated with triggering the destruction of  $\beta$ -cells.<sup>18,68</sup> The only treatment option of T1DM is routine administration of exogenous insulin.<sup>21</sup> A strict insulin regimen must be in place to reduce consequences of hyperglycemia and prevent hypoglycemic episodes.<sup>69</sup> If left untreated, T1DM patients experience wasting, keto-acidosis, and ultimately death.<sup>10</sup>

### **2.1.3 Type 2 Diabetes**

T2DM is a multifactorial, metabolic disease caused by a mixture of genetic and environmental factors.<sup>70,71</sup> Diagnostic criteria of T2DM are the presence of insulin resistance and defective insulin production.<sup>25,60</sup> The synergetic effect of insulin resistance and defects in insulin production cause hyperglycemia leading to detrimental complications.<sup>11</sup> Untreated hyperglycemia may cause life-threatening hyperosmolar syndrome and microvascular disorders.<sup>60</sup> The prevalence of T2DM in the U.S. is a major concern as 18.8 million people were diagnosed in 2012, and 7.0 million were estimated to be undiagnosed.<sup>61</sup> T2DM is the leading diabetic disorder, making up 90-95% of diabetes cases in the United States.<sup>61</sup>

The high prevalence of T2DM is associated with over-nutrition and sedentary lifestyle.<sup>65</sup> Genetic polymorphisms may contribute to the development of diabetes in certain individuals.<sup>70,72</sup> Risk factors of diabetes development include: overweight body mass index (BMI), 19.5-24.9, obese BMI, > 29.9, and insulin resistance.<sup>25</sup> Insulin resistant individuals have ineffective insulin signaling resulting in the reduced uptake of serum glucose into cellular tissue.<sup>25</sup> Pancreatic  $\beta$ -

cells compensate for insulin resistance by increasing insulin production and secretion.<sup>25</sup> Increased serum insulin serves to maintain normal glucose homeostasis, and is common in newly diagnosed diabetic patients.<sup>73</sup> Over time,  $\beta$ -cells dysfunction increases leading to the required treatment of exogenous insulin.<sup>74</sup> T2DM is preventable and can be treated by lowering body weight with a healthy diet and engaging in physical activity.<sup>61</sup>

## **2.2 Obesity**

Obesity is an excess of body weight defined as a BMI greater than 30.<sup>75</sup> Development of obesity is strongly associated with over-nutrition and sedentary lifestyle.<sup>75</sup> In the United States, an alarming 33.4% of the population are estimated to be obese.<sup>76</sup> The rate of obesity and its association with chronic disease is a concern in the medical community. In 2013, the American Medical Association classified obesity as a disease in order to “advance medical intervention for treatment and prevention.”<sup>77</sup> The state of Alabama has a very high rate of obesity, ranking as the 5<sup>th</sup> most obese state with a 33% prevalence rate.<sup>78</sup>

Obesity is associated with several chronic diseases and is an independent risk factor of diabetes mellitus.<sup>79</sup> Obesity is negatively correlated with insulin sensitivity, indicating increased insulin resistance.<sup>25</sup> Excessive fat tissue releases hormonal cytokines which stimulate chronic systemic inflammation and aggravates insulin resistance.<sup>80</sup> Increased circulating free fatty acids in obese individuals are thought to further abrogate insulin action.<sup>81</sup> Obese individuals with visceral fat deposits rather than subcutaneous fat deposits have a greater risk of diabetes development.<sup>26</sup> Reducing body weight restores insulin sensitivity independent of inflammatory markers suggesting excess adipose tissue greatly contributes to insulin resistance.<sup>82</sup>

Childhood obesity is increasing world-wide, although the most recent CDC report states childhood obesity declined from 12% in 2010 to 8% in 2012 within the United States.<sup>78</sup> Obesity in childhood is associated with insulin resistance and arterial plaque, increasing risk diabetes and cardiovascular disease development.<sup>83</sup>

## **2.3 Role of Insulin**

Insulin is a major homeostatic modulator in the body.<sup>84</sup> Insulin serves as a hormonal glucose regulator that acts on muscle, adipose, and hepatic tissue.<sup>30</sup> The pancreas generates insulin from  $\beta$ -cells located within islet granules.<sup>85</sup> Insulin production and release is stimulated in response to increased blood glucose levels or stimulation of intestinal incretin hormones.<sup>19</sup> All islet  $\beta$ -cells simultaneously release insulin into blood circulation upon stimulation of rising glucose levels.<sup>86</sup>

Serum insulin activates cellular insulin receptors (IR), stimulating several cellular signal transduction pathways.<sup>87</sup> Insulin functions as a growth-stimulating hormone, eliciting cell proliferation and inhibiting apoptosis.<sup>87</sup> Postprandial insulin signals adequate nutrition placing the cell in a “fed state,” resulting in induced cellular pathways appropriate in conditions of excess nourishment. Insulin binding with IR stimulates increased glucose uptake, glycogen generation, protein synthesis, and fatty acid synthesis.<sup>30</sup> Insulin stimulation inhibits gluconeogenesis, lipolysis, and glycogenolysis.<sup>30</sup> Insulin stimulates the translocation of the glucose transporter in muscle tissue to the membrane to provide the cell with metabolites of energy production.<sup>30</sup>

Under homeostatic conditions, insulin and glucagon act in concert to regulate blood sugar levels through counter-regulatory mechanisms.<sup>88</sup> When serum glucose levels drop below the threshold (~70mg/dl) glucagon is secreted from pancreatic  $\alpha$ -cells to induce mechanisms active in restoring serum glucose levels.<sup>88</sup> Counter-regulatory mechanisms to insulin action may also be released under stress, illness, or in periods of growth.<sup>89</sup> Insulin antagonists include glucagon, epinephrine, growth hormone, and cortisol.<sup>89</sup>

## **2.4 Insulin Defects in the Diabetic Patient**

### **2.4.1 Insulin Resistance**

Insulin resistance is a multi-factorial condition instigated by sedentary lifestyle, overweight BMI, and high fat, high sugar diets.<sup>5,25,90</sup> Insulin resistance is characterized by abnormal insulin response by skeletal muscle and adipose tissue.<sup>25</sup> Seventy-nine million people in the U.S are affected by insulin resistance as well as millions more world-wide.<sup>6</sup> The presence of insulin resistance is hypothesized to be the underlying cause of T2DM, coronary artery disease, obesity, hypertension, and dyslipidemia.<sup>91</sup> The pathophysiology of insulin resistance remains unclear; however, it is associated with increased BMI, visceral fat mass, and pro-inflammatory cytokines.<sup>91</sup> Inability of insulin to regulate normal cellular function results in increased triglycerides, blood pressure, LDL production, free fatty acids, and gluconeogenesis.<sup>73,92</sup>

Inflammation generated from fat accumulation is considered to be the prominent cause of insulin resistance.<sup>80</sup> White adipose tissue in obese patients secretes pro-inflammatory mediators, TNF- $\alpha$  and IL-6, promoting macrophage infiltration and systemic inflammation.<sup>79</sup> The presence

of systemic inflammation is associated with obesity and insulin resistance.<sup>79</sup> Evidence indicates diacylglycerol (DAG) accumulation in muscle and hepatic tissue enhances insulin resistance suggesting DAG may be an underlying cause of insulin resistance.<sup>93</sup>

Chronic insulin resistance is reversible with diet and exercise.<sup>27,94</sup> Increased exercise intensity is associated with greater insulin sensitivity.<sup>28</sup> Weight loss is directly correlated with increased insulin sensitivity: a study found losing 10% of body weight restores insulin sensitivity.<sup>93</sup>

## **2.4.2 Impaired Insulin Secretion and Signaling**

Symptoms of T2DM impair normal function of protein-protein interactions in the insulin signaling cascade. Furthermore, insulin resistance impedes insulin functioning as a cellular growth hormone and metabolic modulator.<sup>73</sup>

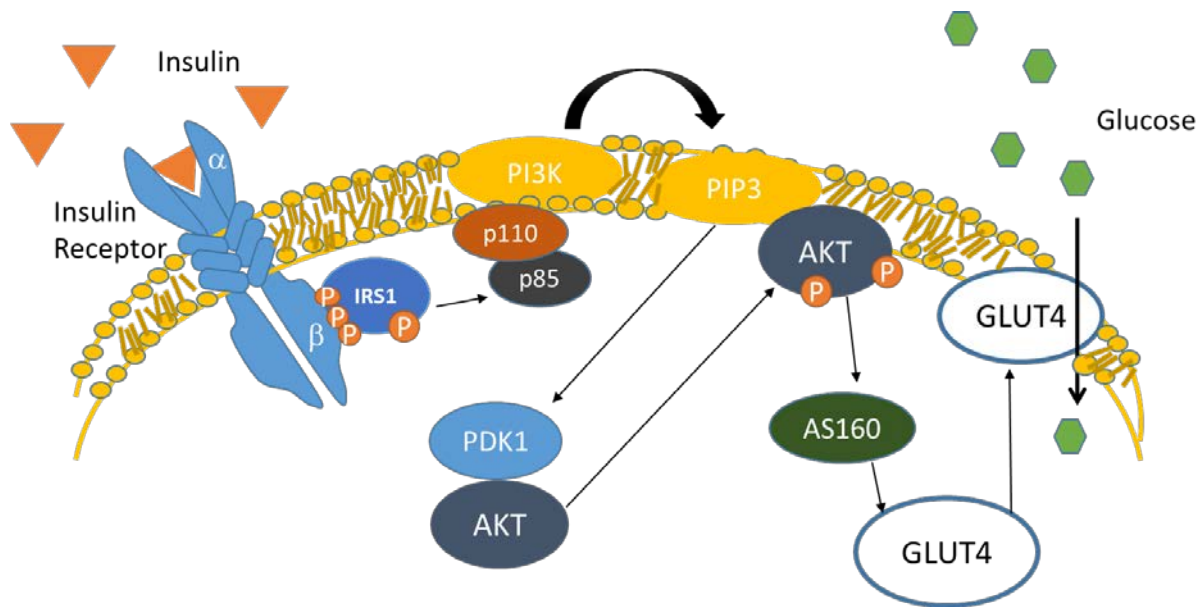
Akt is a major cellular modulator activated by induction of insulin on the insulin receptor.<sup>95</sup> Evidence indicates T2DM pathology impairs normal Akt function.<sup>96</sup> Mature skeletal muscle in insulin resistant ob/ob mice exhibited significantly reduced glucose uptake and Akt phosphorylation (70%) under insulin stimulation.<sup>96</sup> Akt protein expression was reduced in ob/ob mice soleus muscle (25%), liver (25%), and adipose (60%).<sup>96</sup> Declined Akt activation impairs Akt downstream effector inhibition or activation leading to metabolic dysfunction.<sup>32</sup> Insulin resistance in T2DM patients impairs the regulatory feedback system of gluconeogenesis.<sup>73</sup> The gluconeogenic pathway is uninhibited in T2DM causing unregulated production of endogenous glucose resulting in increased serum glucose levels.<sup>73</sup>

Insulin resistance of the skeletal muscle and adipose tissue inhibits glucose uptake into peripheral tissues, and is thought to be the beginning stage of hyperglycemia.<sup>97</sup>  $\beta$ -cells undergo

hypertrophy and hyperplasia facilitating the secretion of abnormally high amounts of insulin (hyperinsulinemia) to maintain normal glucose tolerance.<sup>73</sup> Insulin resistance and hyperinsulinemia precede diabetes and leads to further complications of if left untreated.<sup>91</sup> As  $\beta$ -cell dysfunction progresses, defects to in the first phase insulin response occur followed by  $\beta$ -cell death.<sup>98</sup>

T2DM pathology induces sustained elevated fasting levels of glucose and free fatty acids.<sup>92,97</sup> Elevated levels of glucose and lipids generate toxic conditions aberrant to normal cellular function.<sup>99</sup> Glucotoxicity and lipotoxicity foster sustained ROS levels in  $\beta$ -cells, leading to  $\beta$ -cell apoptosis.<sup>100,101,102</sup> The term glucolipotoxicity has been coined as the presence of both glucotoxic and lipotoxic conditions.<sup>103</sup> Cultured mouse islet cells exposed to glucotoxic conditions (30mM glucose for 72 hours) exhibited significantly reduced glucose stimulated insulin secretion.<sup>104</sup> Lipotoxicity suppresses glucose stimulated insulin secretion and normal insulin function.<sup>105</sup> Increased DAG concentration stimulates serine phosphorylation on insulin receptor substrate 1 (IRS-1), inhibiting PI-3 activation and downstream Akt stimulation.<sup>105</sup>

A small percentage of patients may have insulin resistance due to insulin receptor (IR) defects or defects in the insulin signaling pathway.<sup>106</sup> Hyperinsulinemia results in prolonged insulin stimulation which induces IRS and insulin receptor (IR) degradation, reducing IR concentration, and further impairing normal insulin response.<sup>84,107</sup> Rats with soybean oil induced insulin resistance have decreased expression of GLUT4 and impaired GLUT4 translocation to the plasma membrane suggesting impairment of up-stream signaling events.<sup>108</sup>



## 2.5 The Insulin Signaling Cascade

**Figure 2: The insulin signaling cascade.** Insulin binding to the insulin receptor stimulates auto-phosphorylation of tyrosine amino acids at the  $\beta$  subunit.<sup>109</sup> IRS-1 is phosphorylated and activates the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K). PI3K induces a conformational change of phosphatidylinositol (PI) 4,5-bisphosphate (PIP<sub>2</sub>) to PI 3,4,5-triphosphate (PIP<sub>3</sub>). 3-phosphoinositide-dependent protein kinase 1 (PDK1) is activated and recruits Akt to the cell membrane for phosphorylation. AS160 is activated by Akt and stimulates the translocation of GLUT4 to the cell membrane for glucose uptake.<sup>110</sup>

An elaborate system of mechanisms constitutes the insulin signaling pathway to enhance cellular glucose uptake. Insulin is first secreted from pancreatic  $\beta$  cells located in the islets of Langerhans.<sup>111</sup> Insulin acts on muscle and adipose tissue; stimulating a signaling cascade to ultimately translocate GLUT4 from cytosol to the plasma membrane.<sup>112</sup> Insulin binds to the glycoprotein IR on the cell membrane at one of two extracellular  $\alpha$ - subunits.<sup>113</sup> IR undergoes a conformational change, inducing autophosphorylation of the  $\beta$  subunit.<sup>87</sup>

Upon insulin stimulation, the transmembrane  $\beta$ -subunit of IR is autophosphorylated at Tyr<sup>1158</sup>, Tyr<sup>1162</sup>, and Tyr<sup>1163</sup> and tyrosine kinase is activated.<sup>109,113</sup> Tyrosine kinase then phosphorylates IRS-1 at tyrosine residues.<sup>114</sup> IRS-1 recruits p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K) to the cell membrane.<sup>36</sup> Two Src Homology 2 (SH2) domains of the p85 subunit on PI3K bind with tyrosine receptor domains of IRS-1.<sup>115</sup> IRS-1 binding activates PI3K's 110 catalytic subunit.<sup>114</sup> PI3K activation generates a conformational change in the catalytic domain of PIP2.<sup>40</sup> PIP2 converts to PIP3 which then activates PDK1.<sup>116</sup> Akt is recruited to the membrane and docks with PIP3 through the pleckstrin Homology (PH) domain.<sup>114,117</sup> Docking with PIP3 alters Akt conformation stimulating T<sup>308</sup> and S<sup>473</sup> phosphorylation.<sup>113</sup> Activated Akt continues the downstream signaling cascade resulting in the recruitment of GLUT4 to the plasma membrane.<sup>114</sup> GLUT4 then transports extracellular glucose into the cell for energy production.<sup>118</sup>

## 2.6 Insulin Receptor

Insulin receptors (IR) are tyrosine kinase receptors located within the membranes of mammalian cells.<sup>119</sup> IR are stimulated by insulin and insulin-like growth factors and relay hormonal stimulation via a molecular signaling cascade.<sup>109</sup> IR are highly conserved transmembrane glycoproteins consisting of two  $\alpha$  subunits and two  $\beta$  subunits.<sup>109,119</sup> Extracellular  $\alpha$  subunits function as insulin and insulin-like growth factor binding sites and cellular mediators.<sup>109</sup> Two intra-membrane  $\beta$ -subunits extend into the cytosol and function as an intracellular signal.<sup>120</sup> Upon insulin binding, insulin receptors dimerize into an inter-membrane  $\alpha_2\beta_2$  complex.<sup>113</sup> Dimerization initiates autophosphorylation of both  $\beta$  subunits at Tyr<sup>1158</sup>,



Tyr<sup>1162</sup>, and Tyr<sup>1163</sup>.<sup>35</sup> Phosphorylation of all three tyrosine sites stimulates activation of two intracellular tyrosine kinase subunits.<sup>113,109</sup> Tyrosine kinases elicit IRS-1 phosphorylation at YMXM motifs stimulating the generation of docking sites for SH2 domains.<sup>121</sup> IRS-1 activation is required in the recruitment of GLUT4 to the cell membrane.<sup>121</sup>

Insulin receptors are essential in modulating cellular energy homeostasis.<sup>122</sup> IR knockout mice have rapid onset of insulin resistance, hyperglycemia, hyperinsulinemia, ketoacidosis and death.<sup>122</sup> Patients with mutations in the IR gene exhibit symptoms of severe hyperglycemia and hyperinsulinemia.<sup>123</sup> Tyrosine sites must undergo phosphorylation for signaling, as mutations in the phosphorylation site Lys-1030 inhibit kinase phosphorylation and IR signaling.<sup>124</sup>

Chinese hamster ovary cells (CHO) overexpressing insulin receptors (CHO-IR) or IRS-1 (CHO-IRS-1) are more responsive to insulin stimulation.<sup>115</sup> CHO-IR cells are a prominent model in the investigation of the insulin signaling cascade.<sup>115</sup>

## 2.7 Akt

### 2.7.1 Structure and Isoforms of Akt



**Figure 3: Domains of Akt.** Pleckstrin Homology, kinase, and regulatory domains respectively.

Akt (Protein Kinase B) is a prominent kinase of the AGC kinase family involved in cellular metabolism.<sup>114,125</sup> Three isoforms of Akt are expressed in the body.<sup>114</sup> Akt1 is ubiquitously expressed, Akt2 is found in heart, muscle, kidney, and liver tissue, and Akt3 is found only in the brain and testes.<sup>114</sup> Akt2 is recruited to the plasma membrane upon insulin stimulation and is involved in the regulation of GLUT4.<sup>126</sup>

The N-terminal of Akt is comprised of the highly conserved Pleckstrin Homology domain of about 100 amino acids in length.<sup>127</sup> A kinase domain lays in the central stretch of Akt and a regulatory domain resides at the C-terminal end.<sup>95</sup> The C-terminal has a hydrophobic motif extending 40 amino acid residues in length.<sup>95</sup> The regulatory Thr<sup>308</sup> residue is located in the C-lobe of the kinase domain, and Ser<sup>473</sup> is located within the hydrophobic region of the C-terminal end.<sup>95</sup>

## **2.7.2 Functional Properties of Akt**

The primary function of Akt is modulating cell growth and survival.<sup>39</sup> Akt activation initiates cellular signal cascades involved in nutrient metabolism, cell growth, transcription, angiogenesis, and cell survival.<sup>125</sup> Akt modulates various cellular functions through phosphorylation of target receptor tyrosine kinases.<sup>113</sup> The activating regulatory stimuli of Akt determines Akt's downstream signaling target.<sup>125</sup> Activating stimuli of Akt are diverse and include: basic fibroblast growth factor, insulin, insulin-like growth factor, nerve growth factor, endothelin, interleukins, and TNF- $\alpha$ .<sup>125</sup> Twenty-five up-stream Akt binding proteins have been identified.<sup>128</sup>

Over 170 proteins have been documented as down-stream targets of Akt kinase activity.<sup>129</sup> Akt modulates several pathways of nutrient metabolism through kinase activity of down-stream substrates.<sup>95</sup> Akt activation inhibits glycogen synthase kinase 3 $\beta$  through phosphorylation of S<sup>9</sup>, initiating the de-phosphorylation and activation of glycogen synthase.<sup>113,130</sup> Akt modulates activation of protein synthesis through inhibition of Tsc2.<sup>131</sup> Tsc2 inhibition elicits activation of the mRNA translator protein mammalian target of rapamycin 1 (mTOR1).<sup>131</sup> Akt phosphorylates Foxo1 at S<sup>253</sup> inhibiting hepatic gluconeogenesis.<sup>113</sup> Apart from nutrient metabolism, Akt also serves in the apoptotic pathway through the phosphorylation BAD, inhibiting apoptosis.<sup>39,132</sup>

### **2.7.3 Akt in the Insulin Signaling Cascade**

Upon insulin stimulation, Akt is recruited to the plasma membrane and undergoes phosphorylation at two active sites.<sup>114</sup> The translocation of Akt to the cell membrane is unclear; however, evidence suggests PI3K activates PDK1 which recruits Akt to the membrane through binding with Akt's PH domain.<sup>133,134</sup> Akt docks to PtdIns(3,4,5)P3 (PIP3) and PtdIns(3,4)P2 (PIP2) at the membrane through Akt's PH domain.<sup>135</sup> Lysine 63 (K63) ubiquitin chains associate with Akt facilitating translocation to the plasma membrane.<sup>58</sup> At the membrane, Akt is activated through phosphorylation of Thr<sup>308</sup> by PDK-1, and Ser<sup>473</sup> by the mTORC2 complex.<sup>136,137</sup> Phosphorylation initiates Akt signal transduction through kinase activity.<sup>114</sup> Akt phosphorylates the Rab-GTPase activating protein AS160 (TBC1D4) eliciting the stimulation of GLUT4 recruitment to the plasma membrane.<sup>114,138</sup>

## 2.7.4 Inhibition of Akt

Insulin stimulated Akt activation maintains metabolic homeostasis.<sup>32</sup> The absence or impairment of Akt is clearly associated with the advancement of T2DM.<sup>113</sup> Insulin resistance can impair Akt activation and metabolic regulation, advancing diabetes etiology.<sup>113</sup> Akt KO mice rapidly develop symptoms of T2DM in the absence of active Akt.<sup>113</sup> Inhibition of Akt2 in mature muscle tissue decreased glucose uptake by 70-90%.<sup>96</sup> Akt's upstream signaling protein, PI3K is impaired in type 2 diabetic skeletal muscle abrogating Akt activity.<sup>32</sup>

## 2.8 Sequestosome 1/p62

### 2.8.1 Structure of p62



**Figure 4: Domains structure of p62.** Src homology 2, acidic interaction domain, ZZ finger, TRAF6 binding domain, PEST sequences, LIR motif, and ubiquitin-associated domain respectively.

Sequestosome 1/p62 is a 62 kDa protein with 440 residues involved in modulating cell signaling cascades and protein shuttling.<sup>139</sup> p62 is comprised of eight binding domains: Src homology 2 (SH2), acidic interaction domain (AID/PB1), ZZ finger, TRAF6 binding domain (TRAF6), two proline, glutamatic acid, serine, and threonine (PEST) sequences, a LC3 interacting region (LIR) motif, and ubiquitin-associated domain (UBA) respectively.<sup>140</sup> A Ubiquitin-like Domain (UbL) lays at p62's the N-terminus, SH<sub>2</sub> and AID reside within the UbL domain.<sup>43,141</sup> A ZZ-type zinc finger involved in autophagy lays near the PB1 domain of the N-

terminal.<sup>140,142</sup> The TRAF6 binding domain lays within the center of p62 primary structure.<sup>140</sup> Two PEST sequences lay near the UBA domain at the C-terminal.<sup>140</sup> One LC3 interacting region (LIR) motif involved in targeting autophagy receptor which is between PEST sequences at the C-terminal. Finally, a 48 amino acid Ub-associated domain (UBA) resides at the C-terminal end of p62.<sup>140</sup>

## 2.8.2 Domain Interactions

The binding domains of p62 allow binding with several proteins aiding in the modulation of multiple cell signaling cascades.<sup>141,143</sup> The AID domain at the N-terminal is also known as the PB1 domain.<sup>144</sup> The PB1 domain forms homo and hetero dimers with protein PB1 domains.<sup>144</sup> The PB1 domain serves as a binding site of proteasomes, MAPK kinase, MEK5, MEKK3, and NBR1.<sup>145</sup> The PB1 domain also interacts with the PB1 domain of atypical PKC (aPKC) forming a heterodimer.<sup>141</sup>

The interaction between p62 and ubiquitin involves the UBA domain at the C-terminal.<sup>139</sup> The secondary structure of the UBA domain is a compact three-helix bundle with a hydrophobic surface.<sup>140</sup> The UBA domain of p62 forms a highly stable dimer, enhancing UBA non-covalent binding with K63 and K48 ubiquitin chains.<sup>139,140,146</sup> p62 dimerization drives competition between ubiquitin binding and dimer formation under high concentrations of p62.<sup>139</sup> Low p62 concentrations increases UBA domain affinity for ubiquitin ( $k=40 \mu\text{M}$ ).<sup>139</sup> However, interfering with p62 dimerization decreases NF- $\kappa$ B signaling suggesting dimerization enhances p62 activity.<sup>147</sup> p62 has a higher affinity for polyubiquitinated chains over monomeric ubiquitin and

has a preference for K63 linked chains.<sup>140,146</sup> Deletion of the UBA impairs p62's role in cell survival by impairment of the ubiquitin/proteasome pathway.<sup>146</sup>

### **2.8.3 Functional Properties of p62**

p62 is a signal modulator/adaptor protein involved in receptor-mediated signaling transduction.<sup>43</sup> p62 was first identified as a zeta protein kinase C interacting protein (ZIP homolog) interacting with atypical protein kinase C (aPKC) isoforms through p62's AID motif.<sup>140,148,43</sup> Early studies also lead to p62 identification as a phosphotyrosine independent binding protein with the Src2 domain of p56.<sup>149</sup> Sequestosome 1/p62 is now known as an adaptor protein serving as a modulator in several cell signaling cascades and as a scaffolding protein in protein degradation pathways.<sup>43,146</sup>

#### **2.8.3.1 Adaptor Protein Signal Transduction/Ubiquitination**

p62 is involved in the signal transduction pathways of PKC $\zeta$ , TNF- $\alpha$ , and IL-1 through its role in substrate polyubiquitination.<sup>145,150</sup> Evidence indicates p62 forms a complex with the E3 ligase TRAF6, enhancing substrate polyubiquitination and cellular signaling.<sup>46</sup> The PB1 domains of p62 and MAPK Kinase Kinase (MEKK3) bind, forming a heterodimer.<sup>145</sup> The MEKK3/p62 complex binds with TRAF6, inducing TRAF6 polyubiquitination in the NF- $\kappa$ B pathway.<sup>145,42,46</sup> TRAF6 activation is diminished when p62's UBA, PB1, and TRAF6 domains are deleted suggesting each domain is necessary in TRAF6 activation.<sup>46</sup> Brains of p62 KO mice exhibited no TRAF6 polyubiquitination.<sup>46</sup> Interaction with the ubiquitin ligase, TRAF6, suggests p62 serves in the regulation of K63 polyubiquitination.<sup>46</sup>

Clear evidence indicates the p62/TRAF6 complex is involved in NF- $\kappa$ B, mTORC1 and TrkA activation.<sup>46,151,59</sup> Inhibition of p62 and TRAF6 interaction in PC-12 cells blocked TRAF6 polyubiquitination and inhibited downstream activation of NF- $\kappa$ B.<sup>46</sup> The TRAF6/p62 complex is involved in the K63 polyubiquitination of TrkA, TrkB, and TrkC.<sup>151</sup> The TRAF6/p62 complex is reported to be necessary in the ubiquitination and translocation of mTORC1 to the surface of the lysosome.<sup>59</sup>

### **2.8.3.2 Protein Shuttling/Degradation**

Sequestosome 1/p62 serves as a scaffolding protein in the ubiquitin/proteasome pathway.<sup>146</sup> p62 has been documented to regulate autophagy in various cellular pathways.<sup>142</sup> Mis-folded, or abnormal proteins are tagged with K48 ubiquitin chains by E3 ligases to serve as a signal for degradation.<sup>48</sup> The UBA domain of p62 forms a non-covalent bond with proteins tagged with K48-linked ubiquitin molecules.<sup>152,153</sup> The ZZ-like zinc finger domain and LIR motif also serves as cargo receptors for tagged proteins in the autophagy pathway.<sup>142,154</sup> Sequestosome 1/p62 shuttles tagged proteins and docks to 26S proteasomes with the N-terminal UbL domain<sup>146,155</sup> p62 interaction with a proteasome initiates degradation of tagged proteins.<sup>146</sup>

The role of p62 in NF- $\kappa$ B activation has been extensively studied. NF- $\kappa$ B activity is inhibited when docked to I $\kappa$ B in the cytosol.<sup>156</sup> I $\kappa$ B must be phosphorylated for degradation by two I $\kappa$ B kinases (IKK) leading to NF- $\kappa$ B activation. The TRAF6/p62 complex activates NF- $\kappa$ B through K63 ubiquitination of the IKK complex.<sup>42,157</sup> The activated IKK complex then phosphorylates I $\kappa$ B signaling ubiquitin/proteasome degradation.<sup>145</sup> I $\kappa$ B degradation elicits the

release of NF- $\kappa$ B for translocation into the nucleus for transcription of pro-inflammatory mediators.<sup>156</sup>

#### **2.8.4 Sequestosome 1/p62 in the Insulin Signaling Cascade**

Sequestosome 1/p62 functions as an adaptor protein in the insulin signaling cascade. The SH2 domain of p62 interacts with the YXXM motif of IRS-1 upon insulin stimulation of the insulin receptor.<sup>158</sup> Recent research suggests p62 is necessary for TRAF6 interaction with IRS-1 upon insulin stimulation.<sup>159</sup> p62 forms a homo-dimer at the UBA domain, changing conformational shape to allow docking of dimerized TRAF6.<sup>158</sup> Dimerization of p62 is necessary to adjust to the conformational shape required for TRAF6 activation; deletion of the UBA domain reduces TRAF6 oligomerization and inhibits TRAF6/p62 binding.<sup>46,158</sup> The binding of p62 with TRAF6 generates the TRAF6/p62 complex stimulating TRAF6 auto-ubiquitination and increased ligase activity.<sup>46</sup>

Previous work in our lab demonstrated sequestosome 1/p62 and TRAF6 connects IRS-1 with Akt upon insulin stimulation.<sup>159</sup> Sequestosome 1/p62 knockout and TRAF6 knockout mouse embryonic fibroblasts (MEF) impaired Akt activation.<sup>159</sup> We postulate the TRAF6/p62 complex is required in Akt ubiquitination and recruitment to the plasma membrane. Evidence of insulin-stimulated Akt polyubiquitination through lysine 63 by the E3 ligase TRAF6 has been reported.<sup>58</sup> We hypothesize that Akt serves as a substrate of TRAF6/p62 complex for ubiquitination, translocation to the plasma membrane and down-stream signaling.



## 2.8.6 Function of p62 Relevant to Human Disease

### 2.8.6.1 Obesity and Insulin Resistance

Evidence suggests p62 plays a role in adiposity.<sup>160</sup> p62 knockout mice models develop obesity, glucose intolerance, and leptin resistance and have greater fat deposit in the liver.<sup>161</sup> Gene expression analysis determined excessive weight gain was due to diminished p62 expression.<sup>161</sup> The absence of p62 increased ERK activity thus increasing adipogenesis.<sup>160</sup> sequestosome 1/p62 has been found to play a role in connecting mTORC1 activity to control of adipogenesis.<sup>162,163</sup> Malfunctions in the ubiquitin/proteasome system in the hypothalamus may also contribute to obesity.<sup>164</sup> In the hypothalamus of high fat diet-induced obese Swiss mice co-localization of p62 and ubiquitin were increased and markers of autophagy were significantly reduced.<sup>164</sup> Protein aggregates of p62 and ubiquitin, similar to those found in neurodegenerative disease, increased in the hypothalamus.<sup>164</sup> Inhibition of p62 expression in the hypothalamus resulted in increased body mass.<sup>164</sup>

### 2.8.6.2 Alzheimer's Disease

The regulation of p62 in protein degradation has associated p62 in neurodegenerative diseases.<sup>48,153</sup> p62 serves as a shuttling protein for K63 ubiquitinated proteins tagged for degradation.<sup>146</sup> p62 associates with the 26S subunit of the proteasome resulting in the shuttled protein's degradation.<sup>146,165</sup> Neurodegenerative diseases are associated with inflammation and formation of reactive oxygen species (ROS).<sup>166</sup> ROS causes extensive damage to proteins leading to an increased need for protein degradation.<sup>166</sup> p62 shuttles polyubiquitinated tau and damaged proteins to the proteasome for degradation, thus attenuating Alzheimer's disease

progression.<sup>48</sup> Increased tau phosphorylation has been detected in p62 knockout mice.<sup>167</sup> Active ubiquitin/proteasome pathway improves cell survival and reduces aggregated proteins in the cell.<sup>59</sup> However, over time the ubiquitin/proteasome system malfunctions resulting in aggregations of p62, TRAF6 and ubiquitin in tangles.<sup>146</sup> There is some indication neurodegenerative disease progression may be enhanced in diabetes patients, as p62 expression in the hippocampus and cortex of T2DM rats declined faster with age than controls.<sup>167</sup>

### 2.8.6.3 Paget's Disease of Bone

Sequestosome 1/p62 aberrations are a major component of Paget's disease of bone.<sup>168</sup> p62 regulates osteoclastogenesis and bone homeostasis by acting on receptor activator of nuclear factor  $\kappa$ B (RANK).<sup>168</sup> The loss of p62's C-terminal UBA domain results in abnormal osteoclastogenesis.<sup>169</sup>

## 2.9 TRAF6

### 2.9.1 Structure of TRAF6



**Figure 5: Domains of TRAF6.** TRAF6 is comprised of one RING domain, Z1-Z5 zinc fingers, one coiled coil (CC), and one TRAF-C domain respectively.

Tumor-necrosis-factor-receptor-associated factor 6 (TRAF6) is a 522 amino acid protein comprised of: one Really Interesting New Gene (RING), five TRAF-type zinc fingers, one coiled coil, and a TRAF-C domain from N to C-terminus respectively.<sup>170</sup> The RING-finger domain

located at the N-terminal end of TRAF6 is about 70 amino acid residues in length and consists of eight  $Zn^{2+}$  metal ions bound to Cys, His, and Asp residues forming a cross-linkage.<sup>54,171,172</sup> TRAF6 RING structure at the N-terminal end forms a dimer with TRAF6 adding stability.<sup>171</sup> The TRAF-C domain, also known as the Meprin And TRAF-Homology (MATH), lays at the C-terminus of TRAF6.<sup>54</sup> The TRAF-C domain is made up of 8 anti-parallel  $\beta$ -pleated sheets.<sup>54</sup> The TRAF-C domain of TRAF6 has an unique sequence in its family of proteins and can interact with proteins that other TRAFs are unable to interact with.<sup>54</sup> Some roles of TRAF6 are crucial to survival and loss of TRAF6 cannot be compensated by TRAF family proteins.<sup>51</sup>

## **2.9.2 Functional Properties of TRAF6**

### **2.9.2.1 Ubiquitination**

TRAF6 is an RING-type, E3 ubiquitin ligase and functions as an adaptor protein in cell signaling transductions.<sup>170</sup> Over 70 ubiquitin ligase enzymes have been identified and over 600 RING-domain E3 proteins are found in mammalian cells.<sup>173,171</sup> E3 ligases use the HECT or RING domain positioned at the N-terminus for ligase activity.<sup>172</sup>

Protein ubiquitination by TRAF6 signals protein trafficking and activation.<sup>157</sup> TRAF6 conjugates K63 polyubiquitin chain formation on target proteins.<sup>157</sup> TRAF6 acts in coordination with the E2 enzymes Ubc13 and Uev1A to facilitate K63 ubiquitin chain synthesis.<sup>157</sup> RING and ZZ finger 1 domains of TRAF6 are required for interaction with Ubc13, forming the TRAF6 RZ<sub>1</sub>/Ubc13 complex.<sup>170</sup> Interaction with the E2 enzyme Ubc13 is required in TRAF6 autoubiquitination and IKK polyubiquitination in mouse embryonic fibroblasts.<sup>170</sup>

RING-type E3 ligases are the major E3 form in mammalian cells.<sup>171</sup> Ubiquitin linkage of RING-like E3s may vary, and can be dependent on the association with the bound E2.<sup>171</sup> Dimerization of the N-terminal RING domains of TRAF6 is necessary in promoting TRAF6 ubiquitinating activity.<sup>170,171</sup> Upon dimerization, RING active sites face opposite directions allowing E2 thio-ester binding with TRAF6.<sup>171</sup> The RING finger domain is the binding site of E2 enzymes and the active domain in TRAF6 ubiquitination.<sup>157</sup>

### **2.9.2.2 Pro-inflammatory Mediator**

TRAF6 is an adaptor/modulator protein in the signaling pathway of toll-like receptor (TLR), CD-40, and interleukin-1 (IL-1).<sup>49,53</sup> TRAF6 serves as a downstream effector protein of pro-inflammatory mediators LPS, and IL-1.<sup>51</sup> Upon LPS or IL-1 stimulation, TRAF6 modulates activation of transcription factors of pro-inflammatory cytokines through its involvement in MAPK and NF- $\kappa$ B activation.<sup>174</sup> TRAF6 activates NF- $\kappa$ B through ubiquitination of the docking protein I $\kappa$ B, signaling proteasome degradation. I $\kappa$ B kinase (IKK) activation requires the TRAF6/p62 complex for K63 polyubiquitin in the NF- $\kappa$ B pathway.<sup>157</sup>

TRAF6 KO macrophages exhibited inactive TLR2, 5, 7, and 9, and impaired NF- $\kappa$ B and MAPK activation.<sup>175</sup> TRAF6 deficient cre-loxP mice exhibited inhibited NF- $\kappa$ B signaling, and impaired macrophage expression of IL-10.<sup>176</sup>

Nerve Growth Factor stimulation elicits TRAF6 mediated K63 polyubiquitination of neurotrophin interacting factor and TrkA.<sup>56</sup> TRAF6 is recruited for TrkA receptor internalization from the membrane after the scaffolding protein, p62 associates p75 with TrkA.<sup>56</sup> Blocking CD-

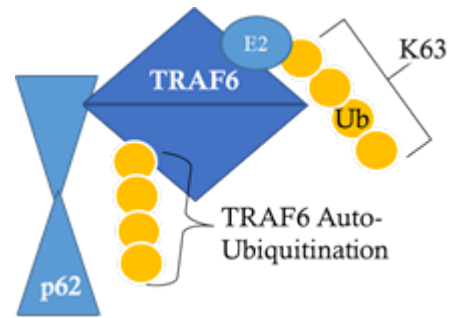
40/TRAF6 interactions in DIO mice attenuated insulin resistance and inflammation, suggesting TRAF6 as a potential therapeutic avenue in insulin resistant patients.<sup>177</sup>

### **2.9.3 TRAF6 in the Insulin Signaling Cascade**

TRAF6 is a E3 ubiquitin ligase serving as a modulator protein in Akt ubiquitination and activation.<sup>58</sup> Upon insulin stimulation, IRS-1 is activated and associates with p62 and TRAF6.<sup>159</sup> Recent evidence from our lab suggests p62 and TRAF6 connect IRS-1 signaling with Akt in insulin transduction.<sup>159</sup> TRAF6 binds at the TRAF6-binding domain of p62 forming the TRAF6/p62 complex.<sup>145</sup> The dimerization of p62 is necessary for TRAF6 activation.<sup>43</sup> TRAF6 and p62 dimers form a TRAF6/p62 complex, eliciting TRAF6 K63-linked auto-ubiquitination at K124 and enhanced ligase activity.<sup>43,52</sup> TRAF6 is reported to be involved in Akt activation and enhanced Akt translocation.<sup>58,178</sup> We hypothesis the TRAF6/p62 complex is required for Akt ubiquitination and recruitment to the plasma membrane and activation.

Growing evidence suggests TRAF6 is involved in Akt activation upon insulin stimulation.<sup>58</sup> Overexpression of TRAF6 in mouse embryonic fibroblasts enhanced Akt translocation to the membrane although, TRAF6 overexpression did not increase Akt binding to PIP3.<sup>58</sup> Depletion of TRAF6 impaired Akt phosphorylation in prostate cancer cells.<sup>58</sup> Akt ubiquitination and phosphorylation at T308 and S473 is decreased in TRAF6 knockout mouse embryonic fibroblasts.<sup>58</sup> p62 knockout MEF cells impaired TRAF6 activation and binding to IRS-1.<sup>159</sup> In hepatic cells, suppressing TRAF6 expression prevents insulin-dependent APPL1 translocation to the membrane, and impairing Akt activation, and insulin-mediated suppression of gluconeogenesis.<sup>178</sup>

**Figure 6: TRAF6/p62 Complex.** TRAF6 and p62 dimers associate forming the TRAF6/p62 complex stimulating TRAF6 auto-ubiquitination and increased ligase activity.

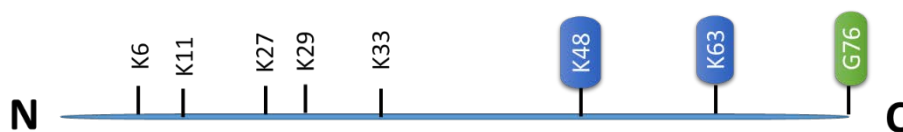


## 2.9.4 Family Proteins of TRAF6

The TRAF family was first identified as Tumor Necrosis Factor (TNF) receptors. There are 6 known TRAF family members: TRAF 1-6.<sup>179</sup> Each TRAF family member serves as a modulator in the TNFR superfamily.<sup>180</sup> The TRAF family is known for the TRAF domain at the C-terminal, also known as the TRAF-C homology (MATH).<sup>180</sup> The TRAF-C domain regulates protein processes and serves in ubiquitin ligase activity.<sup>180</sup> The TRAF-C domain of TRAF6 can bind with XXPXEXX acidic or aromatic consensus binding site.<sup>54</sup> TRAF1 is unique in the family, as it has no RING domain at the N-terminal.<sup>181</sup> TRAF2 is a RING-domain E3 ligase, similar to TRAF6, that mediates K63 polyubiquitination.<sup>182</sup>

## 2.10 Ubiquitin

### 2.10.1 Structure of Ubiquitin



**Figure 7: Active residues of ubiquitin.** Active residues of ubiquitin consist of: K6, K11, K27, K29, K33, K48, K63, and G76.

Ubiquitin is a 76 amino acid polypeptide chain found in all mammalian cells.<sup>183</sup> Only 8.6kDa in weight, ubiquitin binds at other ubiquitin lysine residues forming ubiquitin chains.

All 7 lysine residues of ubiquitin can function as ubiquitin binding sites, indicating 7 types of ubiquitin chain formations.<sup>184</sup> The glycine 76 at tip of the C-terminal end serves as a binding residue to ubiquitinated substrates.<sup>182</sup>

### **2.10.2 Stages of Ubiquitination**

Protein ubiquitination requires three sequential enzymatic reactions.<sup>184</sup> The ubiquitin activating enzyme (E1) initiates protein ubiquitination through an ATP dependent thioester linkage.<sup>171</sup> ATP is used in the ubiquitination process to activate the C terminus of ubiquitin, and promote substrate unfolding and translocation.<sup>172,172</sup> The C-terminus of ubiquitin (G76) is covalently bonded to a cysteine residue of E1.<sup>171</sup> Ubiquitin subsequently forms a thioester linkage with ubiquitin-conjugating enzyme (E2).<sup>171</sup> Ubiquitin protein ligase (E3) interacts with E2 and the protein substrate mediating transfer of ubiquitin to a lysine residue on the target protein or ubiquitin molecule.<sup>171</sup> E3 ligases either catalyze ubiquitination, or facilitate the transfer of ubiquitin chains to the target protein.<sup>171</sup> The C-terminal glycine residue (G76) of ubiquitin binds with a lysine residue on the target protein.<sup>55,185</sup>

Specific E3 ligases function as K48 or K63 ubiquitinating enzymes. Homologous to E6-AP Carboxy Terminus (HECT) E3 ligases accept ubiquitin chains from E2 enzymes and catalyze ubiquitin to substrate proteins.<sup>171</sup> RING-type E3 ligases such as TRAF6 facilitate the transfer of ubiquitin bound with E2 enzymes to substrates.<sup>171</sup> E3 ligases interact with specific target substrates.<sup>153</sup>

### **2.10.3 Functional Properties of Ubiquitin**

Ubiquitin serves as a molecular tag in protein signaling, trafficking, modulation and cell cycle regulation.<sup>183</sup> Ubiquitin chains of four or more ubiquitin bind to a lysine residue of target

proteins to serves as a cellular signal.<sup>185</sup> Polymer chains binding and the conformational linkage of ubiquitin chains is thought to signal the fate of tagged protein.<sup>55,171</sup> Ubiquitin chains linked at lysine K63 are involved in protein trafficking and modulation.<sup>153,186</sup> K48 linked ubiquitin chains signal protein degradation.<sup>153,186</sup> Often, proteins tagged by K48 ubiquitin chains are over-expressed or misfolded proteins targeted for degradation in the ubiquitin-proteasome pathway.<sup>187</sup> K63 linkage is involved in cell signaling, DNA repair, inflammatory activation, and protein trafficking.<sup>185,188,116</sup>

### **2.10.3.1 Protein Modulation/Activation**

Several lines of evidence indicate ubiquitin serves in protein activation.<sup>58,56</sup> Ubiquitination of K63-linked chains elicits activation of the target protein in some cellular pathways. K63 ubiquitination of Akt is reported as necessary for Akt recruitment to the cell membrane.<sup>58</sup> K63 ubiquitination of TrkA elicits TrkA internalization from the cell membrane.<sup>56</sup>

Ubiquitin modulation of the NF- $\kappa$ B and G protein-coupled receptor pathways have been extensively documented.<sup>188,189</sup> TRAF6 is reported in the ubiquitination the interleukin 1 receptor complex (IRAK1), resulting in the recruitment of the IKK complex and TAK1–TAB1–TAB2/3 complex to the membrane in the NF- $\kappa$ B pathway.<sup>188</sup>

### **2.10.3.2 Ubiquitin/Proteasome Pathway**

The ubiquitin-proteasome pathway serves in the degradation of misfolded proteins.<sup>183</sup> K48 ubiquitin tagged proteins are targets of scaffolding proteins such as p62.<sup>43</sup> Scaffolding proteins bind with ubiquitin chains and shuttle proteins to proteasomes for degradation.<sup>43</sup> p62 shuttles K48 tagged proteins to the proteasome and binds with the proteasome at the UbL domain.<sup>43</sup> Proteasomes catalyze proteins to amino acids and small peptides using ATP



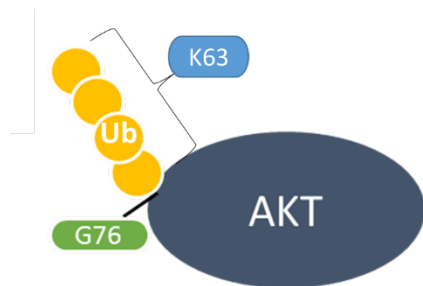
hydrolysis.

Ubiquitination is required in NF- $\kappa$ B activation.<sup>174</sup> K48-linked ubiquitination of I $\kappa$ B signals proteasomal degradation in the NF- $\kappa$ B pathway.<sup>174</sup> Tagged proteins may be spared degradation by de-ubiquitinating enzymes.<sup>183</sup> De-ubiquitinating enzymes release ubiquitin from its substrate before substrate degradation.<sup>183</sup>

#### 2.10.4 Ubiquitin in the Insulin Signaling Pathway

Upon insulin stimulation, p62 interacts with IRS-1 and binds with TRAF6 forming the TRAF6/p62 complex.<sup>46</sup> The binding of p62 with TRAF6 stimulates auto-ubiquitination of TRAF6, activating TRAF6 ubiquitinating activity.<sup>46</sup> As an E3 ligase, TRAF6 is involved in the ubiquitination of target proteins.<sup>157</sup>

Recently in our lab, we found p62 and TRAF6 mediate cellular signaling from IRS-1 to Akt upon insulin stimulation.<sup>159</sup> We postulate, ubiquitination of Akt is required for Akt recruitment to the plasma membrane.



**Figure 8: Lysine 63 Ubiquitination of Akt.** A lysine 63 ubiquitin chain non-covalently binds with Akt at ubiquitin's C-terminal G76 residue.

#### 2.10.5 Ubiquitin Relevant to Human Disease

Malfunctions of the ubiquitin-proteasome pathway has been linked to diseases such as Alzheimer's Disease.<sup>153</sup> Misfolded sAPP $\beta$  proteins involved in plaque formation are degraded by the ubiquitin-proteasome pathway.<sup>48</sup> E3s have several substrate targets and multiple E3s

target the same substrate.<sup>171</sup>

## **2.11 GLUT4**

### **2.11.1 Structure of GLUT4**

GLUT4 is a 510 amino acid protein with 12 helices intersecting through the plasma membrane.<sup>190</sup> GLUT4 generates a hydrophilic tunnel in the plasma membrane for glucose transport.<sup>191</sup> Both the N-terminal and the C-terminal reside within intracellular space.<sup>191</sup>

### **2.11.2 Isomers in the GLUT Family**

The GLUT family is comprised of structurally conserved, facilitative glucose transporters.<sup>190</sup> There are 14 known glucose transporters that are grouped into 3 categories based on their genetic sequence.<sup>192,193</sup> Nearly all mammalian cells express one or more GLUT transporters conducive to the cell's function and environment.<sup>118</sup> GLUT transporters have unique affinities and functionalities enabling GLUT transporters to serve in various cellular environments.<sup>118</sup> GLUT transporters serve in facilitated transport of a variety of saccharide molecules including: fructose, myoinositol, and urate.<sup>192</sup>

The GLUT family may be categorized into three classes.<sup>193</sup> Class one GLUT transporters are the primary transporters of glucose molecules: GLUT 1, 2, 3, and 4.<sup>190</sup> GLUT1 is spread ubiquitously in all cell types and serves in basal glucose uptake.<sup>118</sup> GLUT2 is found in hepatic tissue, kidney tubules, enterocytes, and  $\beta$ -cells.<sup>194</sup> GLUT2 has very low glucose affinity, and requires high glucose concentrations before transport.<sup>194</sup> GLUT3 is a high affinity transporter serving in basal glucose uptake for brain tissue.<sup>194</sup> Class 2 GLUT transporters

primarily facilitate the transport of fructose.<sup>192</sup> The function of class 3 GLUT transporters remains unclear.<sup>192</sup> GLUT4 is known as the primary mediator of insulin-stimulated glucose transport.<sup>195</sup>

### **2.11.3 Activation of GLUT4 and Functional Properties**

GLUT4 is insulin-dependent transporter protein found in muscle, adipose, and hepatic tissue.<sup>194,196</sup> GLUT4 transporters are stored within endosomes and the golgi apparatus under basal conditions.<sup>191</sup> Actions of the insulin signaling pathway stimulate the translocation of GLUT4 to the cell membrane increasing cellular glucose uptake 10-40 fold.<sup>196,197</sup>

The mechanisms of GLUT4 retention in intracellular space and the translocation to the membrane are unclear; however, the Akt substrate, TBC1D4, has been identified as a promising docking protein of GLUT4 within the cytosol.<sup>114</sup> Activated Akt stimulates AS160 activation, which controls Rab10GTPase activation for GLUT4 translocation.<sup>113,198</sup> Inhibition of Akt2 reduced glucose uptake by 70% in mature muscle cells.<sup>96</sup> GLUT4 reportedly interacts with the microtubule network and actin cytoskeleton.<sup>114</sup> Evidence suggests reorganization of the cytoskeleton is required for insulin-stimulated GLUT4 translocation by the Rac1 Rho GTPase signaling arm in mature muscle tissue.<sup>96</sup> After membrane recruitment, the protein GLUT4 storage vesicles (GSV) may play a role in the docking, tethering, fusion, and endocytosis of GLUT4.<sup>114</sup> Apart from insulin, exercise induces insulin-independent GLUT4 translocation to the membrane.<sup>199</sup>

GLUT4 serves as a facilitative glucose transporter with a very high affinity for glucose molecules.<sup>200</sup> GLUT4 also serves in the transmembrane transport of other hexose molecules

including: dehydroascorbic acid (low affinity) and glucosamine (high affinity).<sup>118,201</sup> GLUT4 knockout mice exhibit impaired growth but maintain normal levels of glycemia.<sup>202</sup>

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## **The TRAF6/p62 complex is required for Akt ubiquitination, translocation, and activation.**

### **3.1 Introduction**

Insulin resistance is associated with obesity and is a precursor of diabetes mellitus.<sup>1</sup> Evidence suggests insulin resistance in some individuals may be caused by aberrations in the insulin signaling pathway.<sup>2,3</sup> Akt is a prominent protein in the insulin signaling pathway and is necessary for GLUT4 translocation to the cell membrane in response to insulin.<sup>4,5</sup> Previous research in our lab indicates the proteins p62 and TRAF6 serve in insulin-stimulated Akt activation.<sup>6</sup> Insulin resistance impairs Akt activation and glucose uptake advancing diabetes complications.<sup>7</sup> Further research on protein-protein interactions involved in the insulin-stimulated pathway is needed to understand the molecular mechanisms of insulin resistance.

Insulin binds with the insulin receptor (IR) on the plasma membrane eliciting auto-phosphorylation of tyrosine residues and protein dimerization.<sup>8</sup> Insulin receptor substrate (IRS-1) docked to the insulin receptor is phosphorylated upon dimerization of the insulin receptor.<sup>9,10</sup> The phosphotyrosine motif (YXXM) at the C-terminal end of IRS-1 forms a binding site for Src homology-2 proteins.<sup>11</sup> PI3K docks with IRS-1 inducing a conformational in PIP2 to PIP3.<sup>9,10</sup> PIP3 elicits down-stream signaling activation of Akt.<sup>9,10</sup> PIP3 recruits Akt to the plasma membrane where Akt undergoes phosphorylation at Threonine-308 and Serine-473 by phosphoinositide-dependent kinase 1 (PDK1) and mammalian target of rapamycin complex 2

(mTORC2) respectively.<sup>8</sup> Activated Akt phosphorylates the substrate AS160 (TBC1D4) leading to the recruitment of GLUT4 to the plasma membrane for glucose uptake.<sup>4,9</sup>

Sequestosome 1/p62 is a multi-domain scaffolding protein involved in protein trafficking and signal transduction.<sup>12</sup> Sequestosome 1/p62 binds with the E3 ubiquitin ligase TRAF6 forming the TRAF6/p62 complex.<sup>13</sup> Binding of p62 with TRAF6 induces TRAF6 polyubiquitination, enhances the ligase activity and oligomerization.<sup>14,15</sup>

Recently, in our lab, we identified the YXXM motif of IRS-1 as a docking site for sequestosome 1/p62.<sup>16</sup> Further investigation indicated p62 and TRAF6 associates with IRS-1 and Akt upon insulin stimulation.<sup>6</sup> The absence of p62 or TRAF6 impairs association of Akt with IRS-1 after insulin stimulation.<sup>6</sup> TRAF6, p62, IRS-1, and Akt interact in response to insulin stimulation suggesting p62 and TRAF6 form a bridge between IRS-1 and Akt.<sup>6</sup> TRAF6 is reported to serve as a ligase in the K63 ubiquitination of Akt.<sup>17</sup> Ubiquitin is a 76 amino acids peptide that forms polymer chains which serve as molecular tags in cellular signaling and protein trafficking.<sup>18</sup> In this study, we report ubiquitination of Akt by the p62/TRAF6 complex is necessary for Akt translocation to the plasma membrane and activation.

## **3.2 Materials and Methods**

### **3.2.1 Antibodies and Reagents**

Anti-p62, anti-HA, anti-Myc, anti-ubiquitin, and anti-cadherin were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. TRAF6 antibody was purchased from Abcam, Cambridge, MA. Anti-rabbit IgG and anti-mouse IgG secondary antibody were purchased from GE Healthcare UK Ltd. Enhanced chemiluminescence (ECL) was purchased from Thermo

Scientific. Protein A-Sepharose beads, anti- $\beta$ -actin, anti-tubulin and all other reagents were purchased from Sigma-Aldrich.

### **3.2.2 Cell Culture**

Parental L6 myotubes were incubated at 37°C in DMEM medium supplemented with 2% fetal bovine serum and penicillin/streptomycin. Chinese Hamster Ovary (CHO) cells overexpressing human insulin receptors (CHO/IR) were incubated at 37°C in Ham's F-12 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. DMEM and Ham's F-12 media were purchased from Invitrogen. Wild-type and TRAF6 knockout (TRAF6<sup>-/-</sup>) Mouse Embryonic Fibroblast cells (MEFs) were obtained from Dr. Inoue from the University of Tokyo. Wild-type and p62 knockout (p62<sup>-/-</sup>) MEF cells were obtained from Dr. Ishii from the University of Tsukuba. All cells were grown a humidified incubator containing 5% CO<sub>2</sub> and 95% air at 37°C.

CHO-IR cells were transfected using the cationic lipid method with Lipofectamine<sup>TM</sup> transfection reagent from Invitrogen. Transfected cells were serum starved for 4 hours at 37°C and stimulated with or without insulin (100 nM) for 10 minutes before cell lysis.

### **3.2.3 Immunoprecipitation and Western Blotting Analysis**

Cells were starved in serum free media for 4 hours at 37°C before insulin stimulation. Cells are lysed in Triton lysis buffer (20 mM HEPES (pH 7.6), 150 mM NaCl, 20 mM sodium pyrophosphate, 10 mM NaF, 20 mM 13-glycerophosphate, 1% Triton, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml leupeptin and 10  $\mu$ g/ml aprotinin) to determine protein-

protein interactions. Bradford procedure (Bio-Rad) was used to estimate protein concentration with bovine serum albumin purchased from Sigma Aldrich.

For immunoprecipitation, cell lysates were incubated with 4  $\mu$ g primary antibody. Immunoprecipitates were collected overnight with protein A-Sepharose beads at 4°C. The beads were washed three times with lysis buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to each sample and boiled. Proteins were separated in 10% SDS-PAGE and transferred to PVDF membrane from Millipore. Protein co-interactions were analyzed using Western blotting analysis with appropriate antibodies. Cells were fractionated into cytosol and membrane isolations using ProteoExtract kit from Calbiochem following the manufacturer's protocol.

### **3.3 Results**

#### **3.3.1 Insulin elicits Akt ubiquitination**

Our lab recently found TRAF6 interacts with Akt upon insulin stimulation and K63 ubiquitination of Akt has been reported.<sup>16</sup> We investigated whether Akt is ubiquitinated upon insulin stimulation. L6 myotubes were treated with or without insulin (100 nM) for 15 min at 37°C after 4 hour serum starvation. Cells were lysed with SDS lysis buffer and immunoprecipitated with anti-Akt antibody. Immunoprecipitated samples were immunoblotted with anti-Akt and anti-ubiquitin. Results indicate insulin stimulation induces Akt polyubiquitination. Cells not treated with insulin revealed little or no Akt polyubiquitination (Fig. 9A).

CHO-IR cells were transfected with myc-Akt and HA-ubiquitin plasmids. After 48 hours cells were treated with or without insulin (100 nM) for 10 min at 37° C. Cells were lysed with lysis buffer and protein concentration was determined. Cell lysates were immunoprecipitated with myc and western blotted with HA to detect the tagged Akt ubiquitination and myc antibody. Whole-cell lysates were separated by SDS-PAGE and immunoblotted with anti-myc and anti-HA antibodies to show the constructs were successfully transfected (Fig. 9B). Results provide further indication of Akt ubiquitination only on insulin stimulation.

Insulin resistance leads to impaired Akt signaling in the insulin signaling pathway. To investigate the Akt ubiquitination in diabetic models, control mouse and db/db mouse skeletal muscle tissue was homogenized with lysis buffer. Skeletal muscle homogenates were immunoprecipitated with anti-Akt followed by immunoblotting with Ub and Akt antibodies. Results indicate Akt ubiquitination is impaired in db/db mouse skeletal tissue compared with wild type control tissue (Fig. 9C).

### **3.3.2 Akt is a substrate of TRAF6/p62 complex**

TRAF6 is known to associate with p62 to form the TRAF6/p62 complex.<sup>13</sup> We investigated the interaction of TRAF6 and p62 with Akt in L6 myotubes. L6 myotubes were treated with or without insulin (100 nM) for 15 min at 37°C after 4 hour serum starvation. Cells were lysed with Triton lysis buffer and immunoprecipitated with anti-Akt. Immunoprecipitated samples were immunoblotted with anti-Akt, anti-TRAF6, and anti-p62 antibody. Results indicate p62 and TRAF6 interact with Akt on insulin stimulation. Cells not stimulated with insulin revealed no association between p62, TRAF6, and Akt. The first two lanes are whole cell lysates serving as control samples (Fig. 10A).



The TRAF6/p62 complex serves as an ubiquitinating ligase. We sought to determine if p62 and TRAF6 are necessary for Akt ubiquitination. Wild type and p62 knockout MEFs (p62<sup>-/-</sup>) were stimulated with or without insulin (100 nM) for 10 min at 37°C. Cell lysates were immunoprecipitated with anti-Akt followed by immunoblotting with Akt and Ub antibodies. Cell lysates were western blotted to show endogenous p62 expression of MEFs. Results indicate absence of p62 impaired Akt ubiquitination under insulin stimulation (Fig. 10B). Wild type and TRAF6 knockout Mouse Embryonic Fibroblasts (TRAF6<sup>-/-</sup>) were stimulated with or without insulin (100 nM) for 10 min at 37°C. Cell lysates were immunoprecipitated with anti-Akt followed by immunoblotting with Akt and Ub antibodies. Crude cell lysates were western blotted to show endogenous TRAF6 expression. Results indicate absence TRAF6 impair Akt ubiquitination on insulin stimulation (Fig. 10C). Taken together, our results suggest that both p62 and TRAF6 are necessary for Akt ubiquitination on insulin stimulation.

### **3.3.3 Insulin elicits TRAF6 ubiquitination**

TRAF6 is an E3 ligase associated with K63 Akt ubiquitination.<sup>17</sup> Association with p62 enhances TRAF6 ligase activity, inducing auto-ubiquitination and oligomerization necessary for TRAF6 activation.<sup>13,15,19</sup> To investigate whether TRAF6 gets ubiquitinated on insulin stimulation we used cultures of L6 myotubes.

L6 myotubes were treated with or without insulin (100 nM) for 15 min at 37°C after 4 hour serum starvation. Cells were lysed with SDS lysis buffer and immunoprecipitated with anti-TRAF6 antibody. Immunoprecipitated samples were immunoblotted with anti-Ubiquitin and anti-TRAF6. Results indicate TRAF6 undergoes polyubiquitination on insulin stimulation (Fig 11A).

To determine whether p62 is necessary for TRAF6 ubiquitination in insulin signaling, wild type and p62 knockout MEFS (p62<sup>-/-</sup>) were stimulated with or without insulin (100 nM) for 10 min at 37°C. The lysates were immunoprecipitated with anti-TRAF6 followed by immunoblotting with Ub and TRAF6 antibodies. Cell lysates were western blotted to show endogenous p62 expression. Results indicate absence of p62 impaired TRAF6 ubiquitination on insulin stimulation (Fig. 11B).

To investigate TRAF6 ubiquitination in diabetic models, control and db/db mouse skeletal muscle tissue homogenates were immunoprecipitated with anti-TRAF6 followed by immunoblotting with Ub and TRAF6 antibodies. Results indicate impaired TRAF6 ubiquitination in db/db mice skeletal muscle homogenate compared to control (Fig. 11C). To assess TRAF6 and p62 expression in control and db/db mouse skeletal muscle, homogenates were immunoblotted with TRAF6, p62, and  $\beta$ -actin. Results indicate TRAF6 and p62 expression is decreased in db/db mouse skeletal muscle compared to control (Fig. 11D). Together these results suggest reduced expression of p62 in db/db muscle tissue impairs TRAF6 ubiquitination, thus impairing TRAF6 activation and ligase activity.

#### **3.3.4 TRAF6 and p62 are associated with Akt translocation and activation.**

As TRAF6 and p62 are necessary for Akt ubiquitination, we investigated if TRAF6 and p62 are required for Akt translocation and phosphorylation.

Wild type and TRAF6 knockout MEFs (TRAF6<sup>-/-</sup>) were stimulated with or without insulin for 10 minutes at 37°C. The cytosol and membrane fractions were separated and immunoblotted with cadherin, Akt, and tubulin antibodies. Cadherin antibody was used to verify

membrane fractionation and tubulin antibody was used to verify cytosol fractionation. Results indicate the absence of TRAF6 impairs Akt translocation to the plasma membrane under insulin stimulation (Fig. 12A). Previously in our lab, we found impaired interaction between p62 and Akt in TRAF6<sup>-/-</sup> MEF cells.<sup>6</sup> Taken together, these data suggest TRAF6 interaction with p62 is necessary in Akt translocation.

Wild type and p62 knockout MEFs (p62<sup>-/-</sup>) were stimulated with or without insulin for 10 minutes at 37°C. Samples from the cytosol and membrane were separated and immunoblotted with cadherin, Akt, and tubulin antibodies. Cadherin antibody was used to verify membrane fractionation and tubulin antibody was used to verify cytosol fractionation. Results indicate the absence of p62 or TRAF6 impairs Akt translocation to the cellular membrane on insulin stimulation (Fig. 12B). These results further suggest the TRAF6/p62 complex is necessary in the translocation of Akt.

Wild type, p62 knockout MEFs (p62<sup>-/-</sup>) and TRAF6 knockout MEFs (TRAF6<sup>-/-</sup>) were stimulated with or without insulin for 10 minutes at 37°C. Cell lysates were immunoblotted with phospho Akt (T308), Akt (S473) and non-phospho Akt antibodies. Results indicate phosphorylation of Akt is impaired in the absence of p62 or TRAF6 (Fig. 12C).

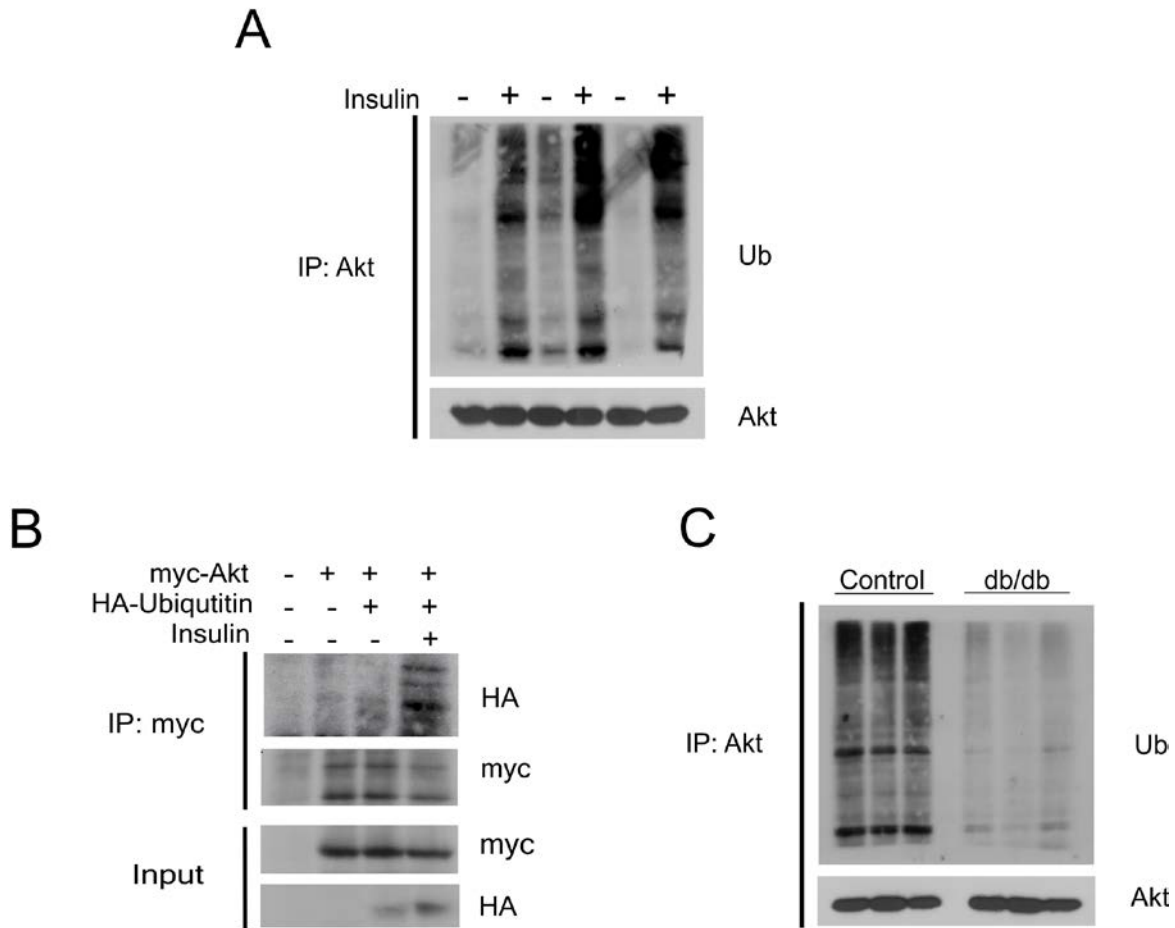
Together, these results indicate the presence of both TRAF6 and p62 are necessary in enhancing Akt ubiquitination, translocation to the plasma membrane and Akt activation. Akt activation is necessary in enhanced glucose uptake through membrane recruitment of GLUT4.<sup>9</sup> Diabetic mouse (db/db) skeletal muscle has declined expression of p62 and TRAF6 impeding Akt ubiquitination necessary for its translocation and Akt activation.

### 3.4 Discussion

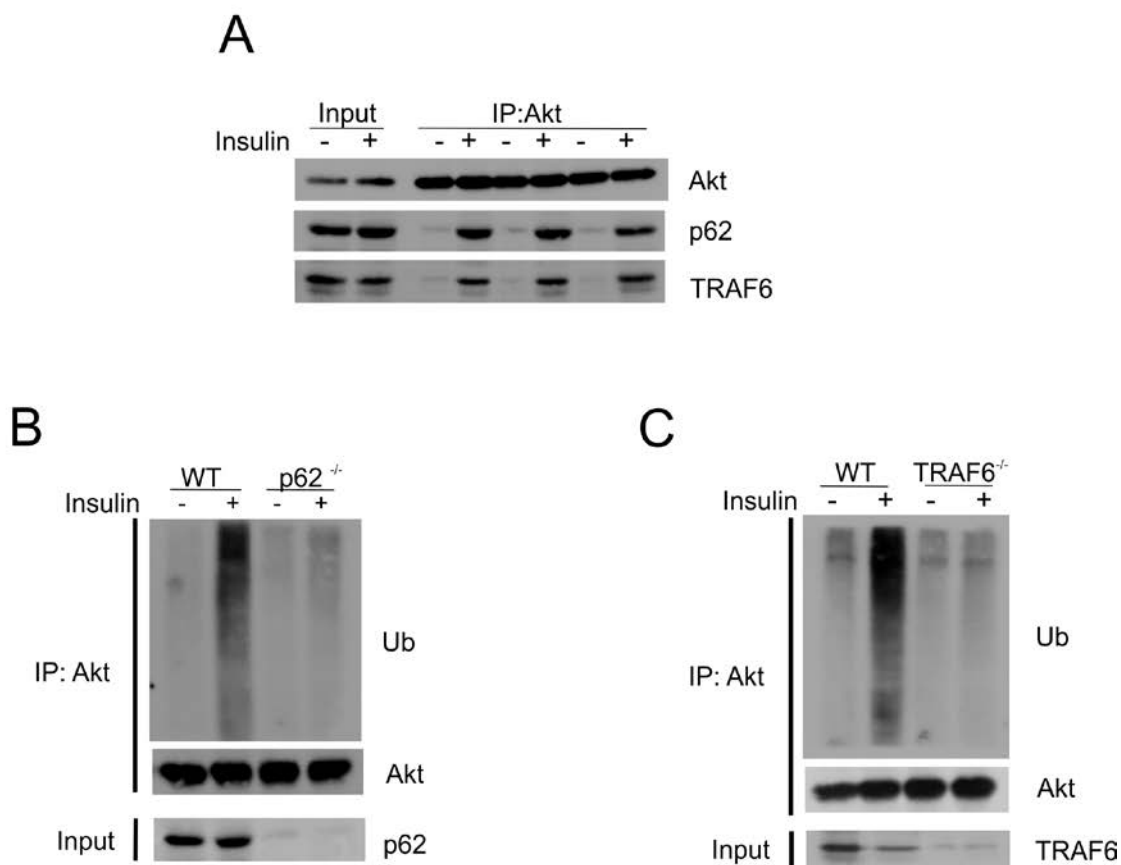
Insulin induces a cellular signaling pathway necessary in the activation of Akt and GLUT4 recruitment to the plasma membrane for glucose uptake.<sup>9</sup> Insulin resistance in diabetes impairs insulin action on the insulin receptor, resulting in declined glucose uptake and abnormal glucose homeostasis. Previous research from our lab has reported interactions of p62 and TRAF6 play a role in enhancing cellular glucose uptake in the insulin signaling pathway.<sup>16</sup>

Our intention was to expand on previous research by furthering investigation on the roles of p62 and TRAF6 in Akt signal transduction. p62 is known to interact with TRAF6 through its TRAF6 interacting domain to form the TRAF6/p62 complex.<sup>20</sup> Akt is found to be a substrate of TRAF6.<sup>17</sup> Activation of Akt is essential in GLUT4 translocation and glucose uptake.<sup>21</sup> p62 serves as a scaffolding protein to enhance the ligase activity of TRAF6.<sup>19</sup> Thus, the E3 ubiquitin ligase activity of TRAF6 may be inhibited by the loss of p62. In this study, we report p62 and TRAF6 form a TRAF6/p62 complex necessary for Akt ubiquitination. The absence of p62 or TRAF6 has been shown to impair Akt translocation and activation. The presence of both p62 and TRAF6 are necessary in Akt ubiquitination, translocation to the plasma membrane, and activation.

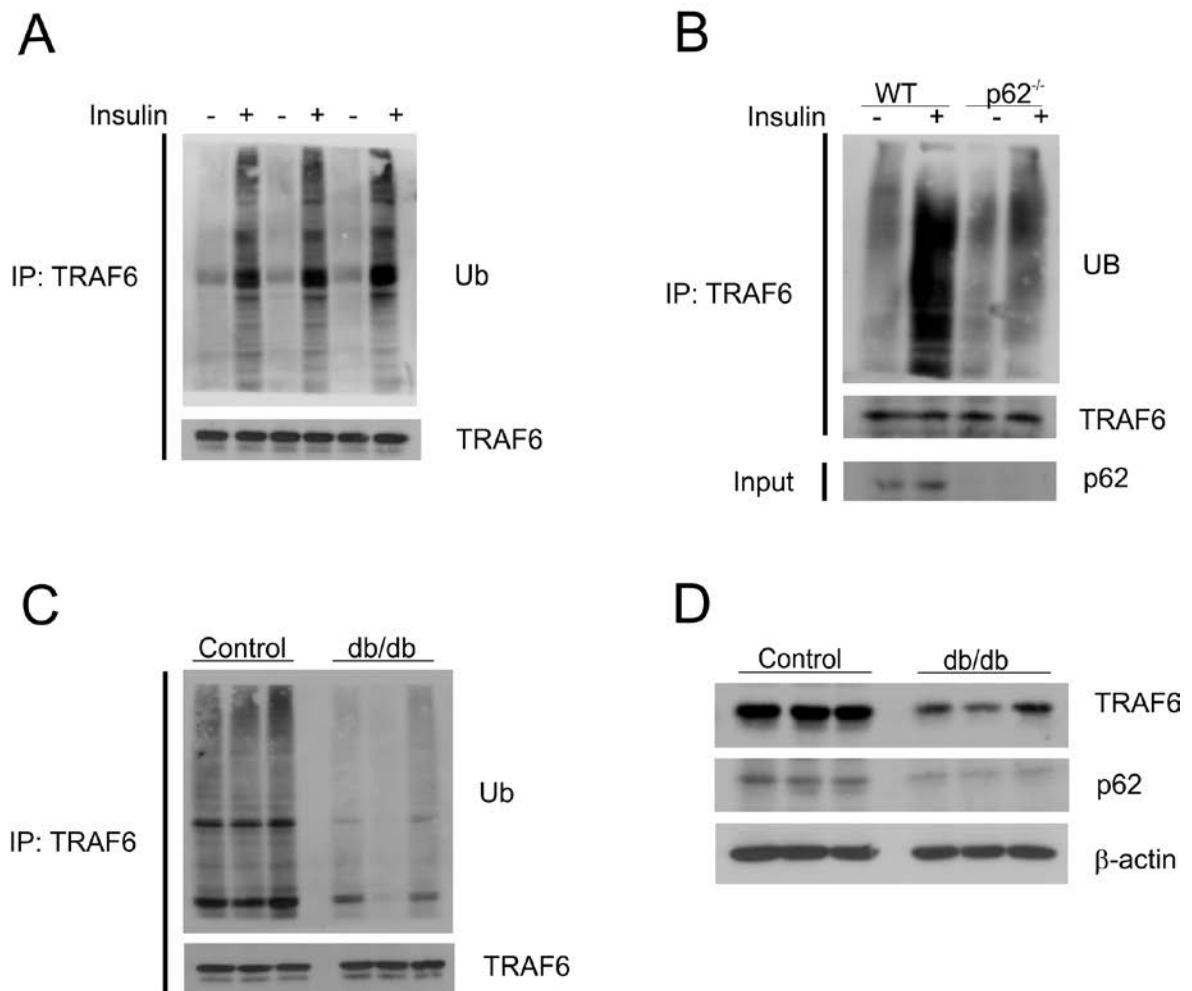
Diabetic mouse (db/db) skeletal muscle have declined p62 and TRAF6 expression and impaired ubiquitination of Akt. TRAF6/p62 complex is necessary for Akt ubiquitination which is required for Akt translocation and activation. These findings reveal p62 and TRAF6 work together for Akt ubiquitination, thus inducing Akt translocation to the plasma membrane and Akt phosphorylation. Deletion of TRAF6/p62 complex affects Akt signaling which may lead to type 2 diabetes.



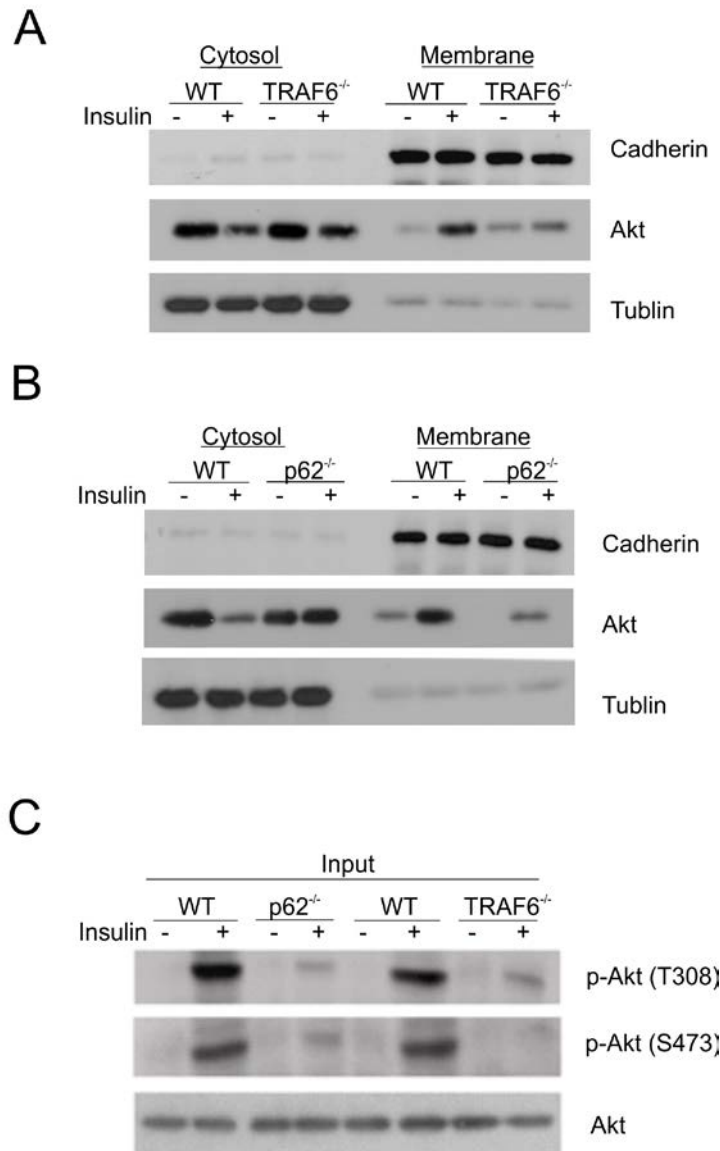
**Figure 9: Akt is ubiquitinated upon insulin stimulation.** A) L6 myotubes were treated with or without insulin (100 nM) for 15 min at 37°C after 4 hour serum starvation. Cells were lysed with SDS lysis buffer and immunoprecipitated with anti-Akt antibody. Immunoprecipitated samples were immunoblotted with anti-ubiquitin antibody and anti-Akt. B) CHO-IR cells were transfected with myc-Akt and HA-ubiquitin. After 48 hours, the cells were treated with or without insulin (100 nM) for 10 min at 37° C. The lysates were immunoprecipitated with myc and HA to detect tagged Akt and ubiquitination. Whole-cell lysates were separated by SDS-PAGE and immunoblotted (WB) with myc and HA antibodies. C) Control mouse and db/db mouse skeletal muscle homogenates were immunoprecipitated with anti-Akt followed by immunoblotting with Ub and Akt antibodies.



**Figure 10: Akt is a substrate of TRAF6/p62 complex** A) L6 myotubes were treated with or without insulin (100 nM) for 15 min at 37°C after 4 hour serum starvation. Cells were lysed with Triton lysis buffer and immunoprecipitated with anti-Akt antibody. Immunoprecipitated samples were immunoblotted with anti-Akt, anti-TRAF6, and anti-p62 antibody. B) Wild type and p62 knockout (p62<sup>-/-</sup>) MEF cells were stimulated with or without insulin (100 nM) for 10 min at 37°C. The lysates were immunoprecipitated with anti-Akt followed by immunoblotting with Ub and Akt antibodies. Cell lysates were western blotted to show endogenous p62 expression of MEF. C) Wild type and TRAF6 knockout (TRAF6<sup>-/-</sup>) MEF cells were stimulated with or without insulin (100 nM) for 10 min at 37°C. The lysates were immunoprecipitated with anti-Akt followed by immunoblotting with Ub and Akt antibodies. Cell lysates were western blotted to show endogenous TRAF6 expression.



**Figure 11: Insulin elicits TRAF6 ubiquitination.** A) L6 myotubes were treated with or without insulin (100 nM) for 15 min at 37°C after 4 hour serum starvation. Cells were lysed with SDS lysis buffer and immunoprecipitated with anti-TRAF6 antibody. Immunoprecipitated samples were immunoblotted with anti-Ubiquitin and anti-TRAF6 antibody. B) Wild type and p62 knockout Mouse Embryonic Fibroblast cells (p62<sup>-/-</sup>) were stimulated with or without insulin (100 nM) for 10 min at 37°C. The lysates were immunoprecipitated with anti-TRAF6 followed by immunoblotting with Akt and Ub antibodies. Cell lysates were western blotted to show endogenous p62 expression. C) Control and db/db mouse muscle tissue lysates were immunoprecipitated with anti-TRAF6 followed by immunoblotting with Ub and TRAF6 antibodies. D) Control and db/db mouse skeletal muscle homogenates were immunoblotted with TRAF6, p62, and β-actin antibodies.



**Figure 12: TRAF6 and p62 are associated with Akt translocation and activation.** A) Wild type and TRAF6 knockout (TRAF6<sup>-/-</sup>) MEF cells were stimulated with or without insulin (100 nM) for 10 minutes at 37°C. Cytosol and membrane fractions were separated and immunoblotted with Akt, tubulin, and cadherin antibodies. B) Wild type and p62 knockout cells were stimulated with or without insulin for 10 minutes at 37°C. Samples from the cytosol and membrane were separated and immunoblotted with Akt, tubulin, and cadherin antibodies. C) Wild type, p62, and TRAF6 knockout cells were stimulated with or without insulin for 10 minutes at 37°C. Samples were immunoblotted with p-Akt (T308), p-Akt (S473), and Akt antibodies.



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