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Novel Protein Phosphatase 2a Complexes In Skeletal Muscle From Obese Insulin Resistant Human Participants

Divyasri Damacharla
Wayne State University,

NOVEL PROTEIN PHOSPHATASE 2A COMPLEXES IN SKELETAL MUSCLE FROM OBESE INSULIN RESISTANT HUMAN PARTICIPANTS

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DIVYASRI DAMACHARLA

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CHAPTER 1 INTRODUCTION

1.1 INTRODUCTION TO DIABETES AND INSULIN SIGNALING PATHWAY

1.1.1 INTRODUCTION TO DIABETES

Diabetes is a metabolic disorder characterized by high blood glucose. According to the National diabetes statistics report, by 2012, 29.1 million of the U.S population had diabetes. Out of which 21 million are diagnosed and 8.1 million undiagnosed. 1.7 million New cases of diabetes have been diagnosed in 2012. 86 million aged 20 or older had pre-diabetes in 2012 compared to 79 million in 2010 which went high by 7 million. In 2010, it was the 7th leading cause of death. There are many complications associated with diabetes besides high blood glucose levels. Eventually, they can have impaired functioning of heart, kidneys, nerves, eyes and blood vessels. These people have higher risk for stroke and heart disease[1]. Neuropathy (nerve damage) is other common effect. This increases the incidence of foot ulcers, infections and also can lead to limb amputations. Diabetic retinopathy associated blindness accounts for 1% of the global blindness[2]. Diabetes can also lead to kidney failure[3].

There are two major types of diabetes: type I and type II. Type II Diabetes accounts for 90-95% of all diabetic cases. Type I Diabetes is seen in children and young adults due to the lack of insulin production caused by the loss of pancreatic beta cells. This is also called insulin dependent diabetes mellitus. Type II Diabetes occurs in relatively older people, which is the consequence of a combination of insulin resistance and relative insulin deficiency[4].

Insulin is a hormone produced by the islet cells in response to the rise in blood glucose levels[4]. More than 70% of insulin-stimulated glucose uptake takes place in

skeletal muscle. Insulin resistance in skeletal muscle is one of the major defects in Type II diabetes[4].

1.1.2 INSULIN SIGNALING PATHWAYS

Insulin increases glucose uptake, synthesis of proteins, lipids and glycogen through activation of various pathways in skeletal muscle cells. Insulin binds to the insulin receptor, a tyrosine kinase receptor, causes the activation of the receptor that triggers cascades of events. The various substrates for the insulin receptor include insulin receptor substrate 1 (IRS1), P60, Gab-1, cbl and Isoforms of shc. The phosphorylated tyrosines on these substrates allow them to bind to various downstream molecules, which include P85 subunit of the phosphatidylinositide 3 kinase (PI3K), Grb2, crk II, etc. PI3K binds to IRS1, which activates Akt to enhance GLUT4 translocation and thereby glucose uptake, glycogen synthesis, and protein synthesis. Insulin also activates mitogen-activated protein kinases (MAPK) to increase gene expression and differentiation[5].

1.2 KINASES AND PHOSPHATASES

Phosphorylation is one of the most important post translational modifications which regulates most signaling molecules in the cell. This process is carried out by kinases contrary to the phosphatases which carry out dephosphorylation. Of all the proteins in the eukaryotic cell, one third portions undergo reversible phosphorylation[6]. Phosphatases remove phosphate group by nucleophilic attack in the presence of water. Phosphorylation and dephosphorylation are mostly carried out on amino acids containing OH group such as serine, threonine and tyrosine. Among these, serine undergoes almost 86.4% of the total phosphorylation followed by threonine (11.8%) and tyrosine

(1.8%)[7]. Kinases are classified as protein tyrosine kinases and serine/threonine kinases. Characterization of phosphatases is based on their substrates:

i. Protein Tyrosine Phosphatases (PTP): remove the phosphate group from phosphorylated tyrosine residues.

ii. Protein Serine/Threonine Phosphatases: remove the phosphate group from phosphorylated serine/threonine residues. This group is Further classified into Phospho Protein Phosphatase (PPP) which includes PP1, PP2A, PP2B, PP4, PP5, PP6, PP7 and Metal ion (Mg^{+2} and Mn^{+2}) dependent phosphatase (PPM) which includes PP2C.

iii. Dual specificity protein serine/threonine/tyrosine phosphatase.

iv. Histidine phosphatase

v. Lipid phosphatase.

As their names suggest, their substrates involve phosphorylated tyrosine, serine/threonine, serine/threonine/tyrosine, or histidine residues and lipids, respectively.

The number of genes coding kinases approximately 3 fold higher than that of phosphatases[8]. Balancing the large number of kinases is achieved by the presence of various isoforms of each subunit composing the phosphatases. Phosphatases are complexes, generally composed of a catalytic subunit and one or more regulatory subunits. The catalytic subunit itself is relatively non-specific and can dephosphorylate numerous substrates. Therefore, the interaction between the catalytic subunit and regulator subunits is required to regulate the specificity and activity of the phosphatases[8].

1.3 PROTEIN PHOSPHATASE 2A (PP2A), REGULATION AND EFFECT OF INSULIN

PP2A is a one of the major serine-threonine protein phosphatase that belongs to the phosphoprotein phosphatase family. This phosphatase constitutes for about 1% of the total protein in the cell[9]. It is a hetero trimeric complex with a dimeric core enzyme (see Figure 2), composed of a 65kda A subunit (PP2Aa), a 55kda B regulatory subunit (PP2Ab), and a 36kda catalytic subunit C (PP2Ac). Subunits A and C form the dimeric core[10].

1.3.1 SUBUNITS OF PP2A

PP2Aa (A regulatory subunit) is ubiquitous and has two isoforms, alpha and beta, which are encoded by two different genes PPP2R1A and PPP2R1B. There is 86% similarity between these two isoforms. The dimer core, in most cases (90%), is composed of A alpha isoform[9]. Both the isoforms are located in the cytoplasm. It is composed of 15 non-identical repeats (HEAT sequence) containing 39 amino acids each. The repeats contain two alpha helices which are connected to each other by intra and inter repeat loops forming a horse-shoe shape. B subunit binds to loops 1-10 whereas C subunit binds to loops 11-15[11]. Because of its flexibility, B subunits and other substrates can be incorporated easily[9]. PP2Aa guides PP2Ac in the interaction with PP2Ab and other substrates and regulates the specificity of PP2Ac[12].

PP2Ab (B regulatory subunit) regulates localization, activity and substrates for the complex. This regulatory subunit is encoded by 15 different genes which are transcribed to a minimum of 26 transcripts and splice variants. They are expressed variably depending on the tissue type. They are classified as B (B55/PR55), B’ (B56/PR61), B‘’ (PR48/PR72/PR130), and B‘’’ (PR93/PR110). They require ATP and Mg^{+2} to be active.

B has four different isoforms and has a tryptophan-aspartate repeat which helps in its identification. B' has five isoforms which are all identical in the center region but different in the C and N terminals. B'' has three isoforms. Different regulatory subunits direct the holoenzyme to perform varied functions. For example, binding of B subunit to the PP2A complex prevents simian virus40 replication whereas binding of B'' does the opposite.

PP2Ac (catalytic subunit) is in globular structure**,** ubiquitously expressed in almost all the tissues and is abundant in heart and brain. **PP2Ac** is conserved from eukaryotes to mammals, with 86% sequence match between yeast and humans. It is responsible for the catalytic activity of the enzyme. **PP2Ac** has two isoforms, alpha and beta, which are 97% identical. Both are composed of 309 amino acids and differ only by 8 amino acids at the N terminal. **PP2Ac** alpha is found mainly in plasma membrane whereas beta isoform is in cytoplasm and nucleus. **PP2Ac** alpha is more abundant than **PP2Ac** beta because of the high degree of mRNA translation[13]. Unique feature of PP2Ac is that C terminal tail is highly conserved (^{304}TPDYEL^{309}). This tail binds to the A and B subunits of the complex.

1.3.2 REGULATION OF PP2A

Given the presence of large number of A, B and C subunit isoforms, various PP2A complexes are possible. The combination of the A, B and C subunit isoforms affects the activity and specificity of PP2A complexes against a particular substrate. Binding and the presence of other regulators can also influence PP2A activity and specificity[14,15].

PP2A activity is also regulated by post-translational modifications[16]. Several experiments in vivo as well as in vitro showed that phosphorylation on Tyr^{307} on $PP2A_c$[16] deactivates $PP2A_c$ by preventing its interaction with the regulatory subunit.

Phosphorylation is also reported in a few PP2A regulatory subunits, which altered their activity and also substrate specificity. In addition, carboxyl methylation on the carboxyl group of the C-terminal residue of Leu^{309} increases the activity of PP2Ac by increasing its binding to A and B subunits. Leucine Carboxyl Methyl Transferase (LCMT), also known as PP2A-Methyl transferase (PPMT), is responsible for methylation of PP2Ac, while PP2A Methyl Esterase (PPME) is responsible for PP2Ac de-methylation.

1.3.3 INHIBITORS OF PP2A

There are two inhibitors which are found to inhibit PP2A which are found through in vitro and in vivo experiments which are termed as I_1^{PP2A} and I_2^{PP2A} [16]. Many small compound found naturally inhibit PP2A. One of them being Okadaic acid which is being used in laboratory practices. It also inhibits other phosphatases like pp1 but at relatively higher concentrations. Other commercially available inhibitors include calyculin a, tautomycin, microcystins, cantharidin and endothall.

1.3.4 ROLE OF PP2A

PP2A is found to be involved in many cell signaling pathways, cell cycle regulation and various other pathways. Experiments conducted by employing phosphatase inhibitor Okadaic acid showed that PP2A plays a role in cell cycle regulation (G2/M transition). Using Yeast, they presented the role of various B subunit analogues in cell cycle, stress response, cytoskeleton organization and morphogenesis. Experiments on drosophila showed the importance of PP2A in early embryogenesis and the changes in the tissue distribution during its development. Several viral antigens are found to interact with PP2A and prevent the inhibitory role of PP2A in those signaling pathways and promote cell proliferation. It is also shown in Xenopus eggs that it involves in initiation

of DNA replication. Several experiments showed the involvement of PP2A in termination of DNA replication, apoptosis, DNA damage response and heat shock response[13].

1.3.5 PP2A IN INSULIN SIGNALING

Saturated fatty acids like palmitate negatively regulate insulin signaling pathway by activating PP2A, which dephosphorylates Akt and ERK1/2. Opposite effect is seen with unsaturated fatty acids like oleic acid or linoleic acid[17].

There are evidences that insulin inactivates PP2A through in vitro and in vivo experiments. Also published evidence shows interaction of PP2A with many signaling molecules, some of which are involved in insulin signaling pathway. Jian Chen et al showed that PP2A is phosphorylated in vitro by the tyrosine kinases which included insulin receptors. It is phosphorylated on Tyr^{307} and this inactivated PP2A[16] .

The effect of insulin on PP2A during myogenesis in rat L6 cells is shown by Srinivasan and Begum. They showed that insulin inactivated PP2A in the differentiated cells .They also showed that the phosphatase activity decreased relatively with the increased concentrations of insulin and also the incubation time[18].

One of the effects of insulin in skeletal muscle cells is the glycogen synthesis through the INS/IRS-1/pkb pathway. Rosanna Cazzolli and associates showed that ceramide treatment of C2C12 skeletal myotubes reduced the glycogen synthesis through inhibition of phosphorylation on PKB upon insulin stimulation. They proved that this inhibition is mediated through activated PP2A via ceramide and thereby effecting the glycogen synthesis in the skeletal muscle cells[19].

It is also shown that PP2A has a positive effect on the INS signaling pathway by preventing the excessive serine phosphorylation on the IRS-1 which will otherwise negatively regulate the pathway. One such serine kinases is ribosomal protein P70 S6K-1 which is an effector of mTOR. They showed direct Interaction of PP2A with IRS-1

in cardiomyocytes protecting IRS-1 from excessive serine phosphorylation. They inferred from their results that PP2A interacts with IRS-1 via mTOR competing for serine residues on IRS-1 and thereby deciding the phosphorylation status of IRS-1. Many factors affect the association, one being the INS stimulation[20].

Only one group conducted experiments on human skeletal muscle where they compared ten type II diabetics with ten controls. They showed that upon insulin stimulation, PP2A levels in control subjects reduced when compared to the basal levels but not in type II diabetics. They also showed corresponding reduction in glucose disposal, glucose oxidation and increase in lipid oxidation[21].

1.4 INSULIN SENSITIVITY; PROTEIN INTERACTIONS AND MASS SPECTROMETRY

1.4.1 METHODS TO MEASURE INSULIN SENSITIVITY

There are several methods to measure insulin sensitivity in humans. Hyperinsulinemic euglycemic clamp and insulin suppression test are used for direct measurement of insulin sensitivity whereas Oral glucose tolerance test and minimal model analysis of frequently sampled intravenous glucose tolerance test are considered for indirect measurement. There are several other Indices used for quick measurement of insulin sensitivity in cases where feasibility is an issue.[22]

For settings where insulin sensitivity measurement and maintenance of steady state conditions is crucial, hyperinsulinemic euglycemic clamp should be the first choice. This technique is also mentioned as a gold standard to assess the action of insulin in vivo[23]. The action of insulin on the body is measured by the rate of exogenous glucose infused to maintain a constant blood glucose concentration. Under conditions of hyperinsulinemia, most (>70%) of the infused glucose is used by skeletal muscle.

This implies that the index measured during the clamp mainly reflects the skeletal muscle sensitivity to insulin[4].

1.4.2 IMPORTANCE OF PROTEIN-PROTEIN INTERACTIONS

Most of the proteins in vivo act in the form of complexes. These proteins contribute to about 80%. Protein interactions play a very crucial role in various functions of the cell, such as gene transcription, signal transduction[24], cell cycle regulation, etc. Correct formation of these complexes is important for the normal body function. Abnormalities in protein-protein interactions cause aberrant cell signals and thereby cause diseases. Many protein complexes have been targeted to treat diseases[25]. Studying the interactions will help us to find out the function of the particular target protein which is specifically useful in cases of any unidentified protein. It will also enable us to analyze the signaling pathways. Protein-protein Interactions have been classified as homo oligomeric /hetero oligomeric based on interaction surface; obligate/non obligate based on stability and transient/permanent depending on persistence[26]

Protein-protein interactions may result in changes in

1. Kinetic characteristics of the complexes
2. Substrate channeling
3. A new binding site on the complex for other effector molecules
4. Substrate specificity
5. Activity of the complex
6. Downstream events

Methods to determine protein-protein interactions. Biophysical methods determine these interactions using the structural information of the proteins. These include X-ray crystallography, NMR Spectroscopy, fluorescence and atomic force microscopy. Direct high throughput methods include yeast two hybrid, affinity purification and mass

spectrometry[27,28,29]. Indirect high throughput methods include gene co-expression and synthetic lethality. Computational predictions of the protein-protein interactions have also been reported[27]. Affinity purification coupled with mass spectrometry (AP-MS) is widely used for identification of interaction networks[28]. Affinity purification allows to enrich the target protein of interest and its co-interaction partners in a single step, and mass spectrometry offers supreme ability to identify proteins from a complex mixture in a high throughout fashion[28,29].

1.4.3 MASS SPECTROMETRY

Mass spectrometry is the most sensitive approach for global identification and quantification of proteins, protein-protein interactions, and protein post translational modifications[30]. The main components of a mass spectrometry instruments, a mass spectrometer, include an ion source, a mass analyzer, and a detector[30]:

1. Ion source: a device to generate charged particles. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are two commonly used ion sources for proteomics studies[30].

2. Mass analyzer: a device to separate the ions based on their mass-to-charge ratio, m/z[30]. Four common types of analyzers for the proteomic analysis include quadruple, Ion trap (quadruple ion trap, linear ion trap), time of flight, and orbitrap analyzers[30].

3. Detector: A detector is a device to record either the charge induced when an ion hits a surface or the current produced when an ion passes by [30]. Two main detectors are electron multiplier (charge induced when ions hit a plate) and image current detector (current produced when ions pass) [30].

As every wet lab experiment, proteomics studies begin with collecting starting material (e.g., tissue, body fluid, cell lysates, etc.) and followed by protein separation

(e.g., affinity capture, electrophoresis, liquid chromatography, etc.). Proteins are cleaved into peptides by enzymatic digestion. The most commonly used protease/enzyme for this purpose is trypsin due to its well defined specificity, which hydrolyzes proteins at the carboxyl side (or "C-terminal side") of the amino acids lysine and arginine. Since one protein may generate many peptides after trypsin digestion, a tryptic digest of a complex mixture of proteins may contain thousands or even millions of peptides. Therefore, the resulting peptides are further separated using a variety of techniques (e.g. affinity capture, liquid chromatography, etc.). The separated peptides are analyzed by mass spectrometry for peptide/protein identification and quantification.

In the present work, the proteomic approach developed in our laboratory[31] was applied to investigate PP2Ac interaction partners in human skeletal muscle from overweight/obese non-diabetic human participants. I asked following three questions 1). Are PP2Ac interaction partners reported in cell culture or animal models indeed interact with PP2Ac in human skeletal muscle? 2). Are there new PP2Ac interaction partners in human skeletal muscle? 3). Are there insulin responsive PP2Ac interaction partners in human skeletal muscle?

CHAPTER 2 RESEARCH DESIGN AND METHODS

Clinical and proteomics studies were carried out similar to those describe[31], which reported the discovery of new IRS1 interaction partners in human skeletal muscle. The main difference was that PP2Ac Co-immunoprecipitation was used to enrich PP2Ac interaction partners in the present work instead of IRS1 Co-immunoprecipitation used in the publication.

As illustrated in Fig. 3, the approach we used included extensive clinical and proteomics data acquisition and data analysis. We first recruited subjects which was followed by comprehensive tests to screen them for eligibility. This is followed by hyperinsulinemic-euglycemic clamp, procedure to measure insulin sensitivity and muscle biopsies are collected. The study was performed in the following order: biopsy homogenization; immunoprecipitation of the "bait" protein (PP2Ac), at the endogenous level; followed by one dimensional SDS-PAGE to separate co-interaction proteins; in-gel trypsin digestion to generate peptide fragments; and HPLC-ESI-MS/MS analysis to identify co-immunoprecipitating proteins. Multiple biological comparisons and immunoprecipitation of NIgG (as non-specific control) were used for false positive minimization. Extensive literature searches as well as bioinformatics were used to integrate clinical and proteomics data and to identify pathways and functional categories in which identified PP2Ac interaction partners were involved.

2.1 MATERIALS

2.1.1 REAGENTS

Reagents are from these suppliers: sequencing-grade modified trypsin (Promega, Madison, WI); protein A sepharose and iodoacetamide (Sigma, St Louis,

MO); C18 ZipTip (Millipore, Billerica, MA); antibody to PP2Ac (Upstate/Millipore, Billerica, MA).

2.1.2 SUBJECTS

A total of 8 overweight/obese non-diabetic volunteers were recruited and took part in the study at the C. S. MOTT Clinical Research Center at Wayne State University. Written consent was attained from all participants and the study was explained in detail including the indirect benefits and risks. No one had any significant medical problems, and none engaged in any heavy exercise, and they were directed to stop all kinds of exercise for at least 2 days prior to the study. Institutional Review Board of Wayne State University approved this protocol.

2.1.3 HYPERINSULINEMIC-EUGLYCEMIC CLAMP WITH MUSCLE BIOPSIES

A hyperinsulinemic-euglycemic clamp was used to assess insulin sensitivity and expose skeletal muscle to insulin *in vivo*, as previously described[31]. Followed by a ten hour overnight fast, the study began at approximately 08:30 hours (time -60 min).Two catheters were placed, one in an antecubital vein, maintained throughout the study for infusions of insulin and glucose. The second in a vein in the contra lateral arm, which was covered with a heating pad (60°C). The purpose of heating pad is to arterialize the venous blood being collected. Blood samples were collected for determination of plasma glucose concentrations. At approximately 09:00 hours (time −30 min), under local anesthesia, a percutaneous needle biopsy of the vastus lateralis muscle was performed. These biopsy samples were blotted free of blood, cleaned of connective tissue and fat (~30 sec), and then frozen in liquid nitrogen. At 09:30 hours (time 0 min), continuous human insulin (Humulin R; Eli Lilly, Indianapolis, IN) infusion was begun

at a rate of 80 mU m^{-2} $minute^{-1}$, and continued for 120 min. Plasma glucose was measured at 5-min intervals throughout the clamp. Euglycemia was maintained at 90 mg/dl by variable infusion of 20% d-glucose. Another biopsy is taken at 11:30 hours (time 120 minutes) in the contralateral leg.

Plasma insulin concentration was calculated using the ALPCO Insulin ELISA Jumbo (Alpco Diagnostics, Salem, NH).

2.2 PROTEOMICS SAMPLE PREPARATION AND ANALYSIS

2.2.1 SAMPLE PREPARATION

Biopsies were homogenized and processed as described[31-33]. The lysate proteins were precleared with NIgG followed by PP2AC immunoprecipitation. The co-immunoprecipitates were resolved on one dimensional SDS-PAGE, which is followed by in-gel trypsin digestion, peptide enrichment, and HPLC-ESI-MS/MS analysis using a LTQ-Orbitrap Elite as described[31]. Peptides/protein identification and quantification were performed using the MaxQuant software. It is one of the most prevalent quantitative proteomics software[34]. Using this, peak areas for each protein were obtained by selecting the option for label-free quantification (LFQ). Only those proteins with a minimum of 2 unique peptides and with false discovery rate (FDR) at 0.01 were considered. In total, 2057 proteins were identified in the 16 muscle biopsies using HPLC-ESI-MS/MS.

To be considered as a PP2Ac interaction partner, a protein has to additionally pass these following criteria: 1). with an enrichment ratio >10; 2). Identified with LFQ peak area (PA) in more than half of the PP2Ac IP (i.e. >8 biopsies used). The enrichment ratio was calculated as follows: 1st, PA for a protein identified in a gel lane was

normalized against the sum of the peak areas for all proteins identified in the same gel lane to obtain normalized ratio for individual protein, Norm:*i*,

$$\text{Norm:}\, i = \frac{\text{PA}i}{\sum_1^n \text{PA}i}$$

Then, the average of normalized ratio for each protein in the IRS1 co-immunoprecipitates, Average_Norm:*i*_IRS1, as well as the average of normalized ratio for the same protein in the NIgG co-immunoprecipitates, Average_Norm:*i*_NIgG, were obtained. Finally, Average_Norm:*i*_PP2Ac was divided by Average_Norm:*i*_NIgG, which gives the enrichment ratio for each protein.

$$\text{Enrichment\_Ratio:}\, i = \frac{\text{Average\_Norm:}\, i\text{\_PP2Ac}}{\text{Average\_Norm:}\, i\text{\_NIgG}}$$

Proteins exclusively detected in the PP2Ac immunoprecipitates were identified as we used NIgG as a control. Nevertheless, this will give rise to false negatives. But due to the high sensitivity of our method, trace amounts of a protein will be detected even it was non-specifically absorbed on the NIgG beads, it may be identified with minimum 2 unique peptides with FDR at 0.01. However, if a protein is true component of the PP2Ac complex, higher peak area will be assigned to this protein in the PP2Ac sample than in the NIgG sample.

To determine the relative quantities of PP2Ac interaction partners in human skeletal muscle biopsies before and after insulin infusion in vivo, the PA for each protein identified in a specific biopsy was normalized against the PA for PP2Ac identified in the same biopsy, which results in Norm:*j*.

$$\text{Norm:}\, j = \frac{\text{PA}j}{\text{PA\_PP2Ac}}$$

The normalization strategy is widely used in proteomics studies involving protein-protein interactions[35], and uses similar concept as in western blotting, where the signal for an interaction protein is normalized against that for the protein serving as the

"bait." The normalized peak area for each PP2Ac interaction partner, Norm:*j*, was converted to log2 form and compared within the group to assess effects of insulin on protein-protein interactions involving PP2Ac.

2.2.2 STATISTICAL ANALYSIS

Although a large number of proteins were assigned in at least one of 16 biopsies that were studied, various filters narrowed the number of proteins that were used in comparisons among groups as described above. This approach is diagrammed in Fig. 5. To assess the effects of insulin within group, statistical significance was calculated applying paired *t* tests. Changes were statistically significant at $p<0.05$.

Pathway analysis on PP2Ac interaction partners was performed using Ingenuity Pathway Analysis (Ingenuity Systems, Inc., Redwood City, CA), which is widely used and contain biological and chemical interactions and functional annotations created by manual curation of the scientific literature[36]. A pathway was considered significantly enriched if the p-value for that pathway was less than 0.01 and contained at least 4 identified PP2Ac partners.

CHAPTER 3 RESULTS

Clinical characteristics of the eight overweight/obese control subjects. Table 4 lists the clinical characteristics for the subjects.

3.1 PP2AC INTERACTION PARTNERS IN SKELETAL MUSCLE FROM OVERWEIGHT/OBESE HUMAN PARTICIPANTS

PP2Acα and PP2Acβ were detected in PP2Ac immunoprecipitates from all 16 biopsies used for the study, but were not detected at all in the NIgG immunoprecipitates. In total, 186 proteins met the criteria for classification as PP2Ac interaction partners (Figure 5 and Table 4). Note that PP2Ac interaction partners listed in Table 5 may involve both direct and indirect partners.

Table 4 lists the 186 PP2Ac interaction partners with fold changes between biopsies obtained before and after insulin infusion in overweight/obese human participants. Among these 186 PP2Ac interaction partners, 14 partners were previously reported while 172 were novel. The known PP2Ac interaction partners included AMPK, CCT2, CCT3, CCT4, CCT5, CCT6A, CCT8, IGBP1, PPME1, PPP2R1A, PPP2R2A, Rpn2, TCP1, TUBA8.

Ingenuity pathway analysis on the 186 PP2Ac interaction partners and PP2Ac suggested various pathways significantly enriched compared to the whole genome background, such as mTOR and MAPK signaling (Figure 6 and Table 5). One of the significantly enriched pathway, mTOR signaling, was illustrated in Figure 7

We also performed network analysis using Ingenuity pathway analysis for the 186 PP2Ac interaction partners and PP2Ac to illustrate how these partners can be interrelated. Figure 6 shows the network with the highest score and highest number of interaction partners identified in this study.

3.2 INSULIN-RESPONSIVE PP2AC INTERACTION PARTNERS IN SKELETAL MUSCLE FROM OVERWEIGHT/OBESE HUMAN PARTICIPANTS

Upon insulin infusion in vivo, 17 PP2Ac interaction partners showed significant difference in their association with PP2Ac ($P<0.05$). Among them, ten PP2Ac interaction partners showed decreased interaction (Table 6) and the remaining seven showed increased interaction (Table 7).

CHAPTER 4 DISCUSSION

4.1 PP2AC INTERACTION PARTNERS IN SKELETAL MUSCLE FROM OVERWEIGHT/OBESE HUMAN PARTICIPANTS

Using the proteomics approach for protein-protein interactions developed in our laboratory[31], we have identified 186 PP2Ac interaction partners in skeletal muscle form 8 overweight/obese human participants, which represents the largest PP2Ac interaction network in humans to date. Among them, 14 were known PP2Ac interaction partners while 172 were novel (Table 4).

Among these 14 PP2Ac interaction partners, PPP2R1A and PPP2R2A are 'A' subunit alpha isoform and 'B' subunit alpha isoform of the PP2A complex, respectively. PPME1 is protein methylesterase, which catalyzes the demethylation of PP2A on leucine309. As mentioned in the Introduction, demethylation of the catalytic subunit at this site may decrease the stability of the PP2Ac complex and subsequent PP2A activity. When PP2Ac is not exit as a complex, it will be directed for proteasome degradation by the E3 ubiquitin ligase. IGBP1 also known as alpha4, binds to catalytic subunit thereby stabilizing and preventing it from the degradation[37]. CCT2, CCT3, CCT4, CCT5, CCT6A, CCT8, TCP1 are chaperone proteins. All the translated proteins need to be folded properly in order to achieve the tertiary structure. Sometimes, they can be misfolded. These misfolded or unfolded proteins can aggregate to form lethal complexes. The function of these chaperones is to correct the partially folded or misfolded proteins using ATP as source of energy[38]. Rpn2 is Ribophorin II, which is subunit of the enzyme oligosaccharyltransferase complex. This enzyme transfers glycosyl group from a lipid molecule to asparagine of Asn-x-Thr/Ser sequence. The enzyme is specific to the membrane of Rough endoplasmic reticulum. Glycosylation is a post translational

modification of the proteins. This modification of nascent proteins is important sometimes in the proper folding of the proteins[39]. TUBA8 belongs to alpha tubulin protein family that heterodimerizes and forms microtubule[40]. AMPK is a protein kinase which is activated in response to altered energy levels in the cell. PP2A has been shown to be able to dephosphorylate AMPK[41]. Higher ATP levels reduce the activity of AMPK. When the AMP levels rise, ATP is exchanged for AMP and this activates AMPK. AMPK comprises of alpha beta and gamma subunits. Alpha and beta having two isoforms and gamma subunit with three isoforms. Identified isoforms are PRKAG1 (gamma 1), PRKAA2 (alpha 2), PRKAB2 (beta 2). PRKAG1 is ubiquitously expressed whereas PRKAA2 and PRKAB2 are found abundant in skeletal muscle cells. It is known to have important role in skeletal muscle insulin sensitivity[42]. In skeletal muscle, activation of AMPK will cause fatty acid and glucose oxidation. It also plays a role in activation of GLUT4 transporters which are responsible for uptake of glucose upon recruitment to plasma membrane. Plays a role in glycogen metabolism[43]. Activation is carried out by phosphorylation of AMPK at various serine and threonine sites. It is phosphorylated by minimum three kinases. Its binding to PP2Ac may change the phosphorylation status of this kinase and thereby its activation or inactivation. Since denovo AMP synthesis will activate AMPK, experiments were conducted using rat hepatocytes to see if altering the activity of enzymes involved in purine biosynthesis will improve insulin sensitivity. They found that abundant adenosuccinate lyase (ADSL) can lead to increased AMP production. There by AMPK activation and improved insulin sensitivity[44]. Both ADSL and AMPK are found as potential interaction partners.

mTOR pathway has great impact on the cell growth and metabolism. It regulates protein biosynthesis, lipid synthesis, mitochondrial biogenesis and metabolism. Previous reports show that PP2A is down regulated by mTOR, and degradation of IRS1 by

mTOR is achieved through inhibition of PP2A[45]. In the present work, we detected transcription factors (elf3 and elf4G) that regulate protein synthesis as partners of PP2Ac. Growth factors like insulin stimulate mTOR by increased phosphorylation of TSC2 protein by kinases like PKB, ERK1/2 and RSK1. This TSC2 phosphorylation leads to inactivation of TSC1/2 and there by activation of Mtor. AMPK is activated in response to low energy levels. This activated AMPK phosphorylates and reduce the activity of TSC2 and thereby reduce mTOR activation[46]. RSK1 and AMPK regulate the mTOR pathway mainly by phosphorylation events by kinases and dephosphorylation events possibly regulated by PP2A.

4.2 INSULIN-RESPONSIVE PP2AC INTERACTION PARTNERS IN SKELETAL MUSCLE FROM OVERWEIGHT/OBESE HUMAN PARTICIPANTS

Upon insulin infusion in vivo, 10 PP2Ac interaction partners had a decreased association with PP2Ac ($P<0.05$, Table 6). Among them, 5 were involved in vesicle traffic, including Archain1, Adp-ribosylation factor 4, Coatomer protein complex subunit alpha, Sec1 family domain containing 1, Vesicle amine transport 1. Archain1, also known as coatomer protein complex subunit delta, and COPA, which is coatomer protein complex subunit alpha, are coat proteins. They have important function in transporting proteins between Golgi apparatus and Endoplasmic reticulum. They are also involved in budding of the vesicles from the Golgi membrane. These coat proteins require ARF proteins for this function[47]. ARF proteins are GTP-binding proteins which upon activation at the membrane recruit these coat proteins and thereby aiding these vesicles to bud from Golgi apparatus[48]. ARF4 is involved in insulin mediated activation of Phospholipase D and also Vesicle trafficking. SCFD1, Sec1 family domain containing 1, is also involved in vesicle transport between Golgi and Endoplasmic

reticulum. VAT1, vesicle amine transport 1, is found to be involved in regulation of mitochondrial fusion. It is shown to have ATPase activity.

Glycogenin is a primer for the glycogen synthesis which is further extended by glycogen synthase and glycogen branching enzyme[49].

Proteasome 26S subunit, ATPase, 3 is part of the proteasome complex which degrades proteins that are tagged with ubiquitin[50]. Ubiquitin proteasome plays an important role in regulation of Insulin signaling pathway by ubiquitination and subsequent degradation of Insulin Receptor Substrate, GLUT1 and GLUT4[51].

STAT3 is a transcription factor which upon activation by any growth factor will cause transcription of appropriate genes. It was showed that phosphorylated STAT3 amounts are increased by two fold in overweight T2D compared to overweight controls. STAT3 is also shown to contribute to insulin resistance in various tissues like liver and smooth muscle[52].

Upon insulin infusion in vivo, 7 PP2Ac interaction partners had an increased association with PP2Ac ($P<0.05$, Table 6). ATP synthase beta polypeptide and dihydrolipogenase are mitochondrial proteins. ATP synthase beta is one of the subunits of the catalytic core of ATP synthase enzyme. This enzyme catalyzes the formation of ATP from ADP in the mitochondria[53]. Mitochondrial dysfunction is linked to obesity and Type II Diabetes. Experiments were conducted on human skeletal muscle comparing the phosphorylation of this protein between basal and insulin stimulated biopsies of lean, obese and T2D. The amount of ATP synthase in basal biopsies is found to be decreased in obese and T2D compared to lean. They found abnormal phosphorylation sites in obese and T2D[54]. As a partner of PP2Ac, It is possible that phosphorylation and dephosphorylation of this protein is regulated by PP2A which thereby influences Insulin signaling and insulin resistance.

PGM1, phosphoglucomutase-1, is involved in the breakdown and formation of glucose, Conversion of glucose 6 phosphate to glucose 1 phosphate and vice versa[55].

NT5C3, 5' Nucleotidase, breaks down nucleotides to nucleosides and phosphate.

ADSL, Adenylsuccinate lyase, catalyzes two steps in de novo Adenosine Mono Phosphate synthesis.

PPP1R7 is a regulatory subunit of protein phosphatase 1 (PP1). PP1 also belongs to phosphoprotein phosphatase family, and is also a serine/threonine protein phosphatase[56].

PPP2R2A is one of the regulatory subunits of PP2A. Binding of this subunit catalyzed many dephosphorylation events involved in cell cycle regulation[57], DNA repair[58], TGFβ signaling[59], Wnt signaling[60] and diseases like cancer. In islet β cells, presence of this subunit directed PP2A to dephosphorylate FOXO1 under oxidative stress conditions[61]. Considering the role of regulatory subunits in regulating the localization and specificity of PP2A, unveiling its role in skeletal muscle may reveal important information in specific to skeletal muscle.

4.3 SUMMARY

In the present work, PP2Ac was found to interact with 186 protein partners either directly or indirectly in human skeletal muscle. This is the largest PP2Ac interaction partner network found in humans till date. Moreover, seventeen insulin-responsive PP2Ac interaction partners in skeletal muscle were identified in overweeight/obesse human subjects. Validating some of the important partners (partners that may directly or indirectly effect the insulin signaling pathway) would be the next step. In addition, comparison with lean and T2D participants will discover the differences in the PP2Ac partners among the 3 groups. This information will help us understand the role of PP2A in the development of inuslin resistance and T2D.

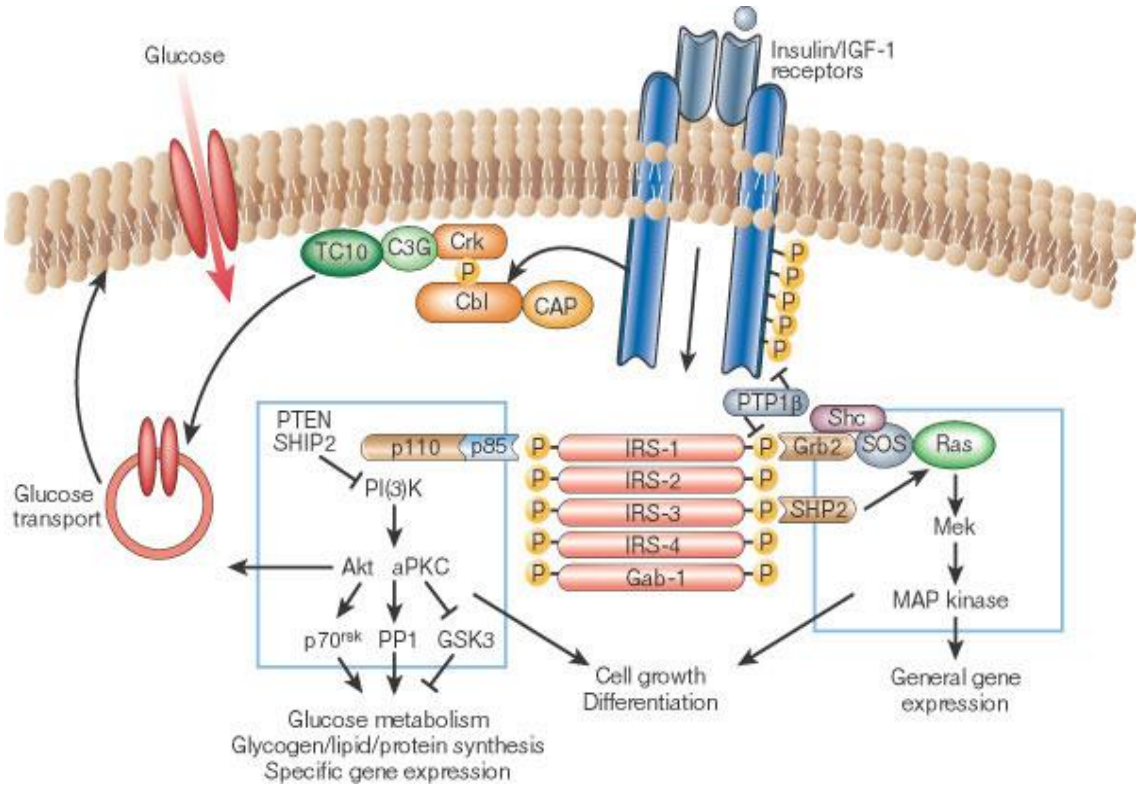

Figure 1. Insulin signaling pathway showing the signaling molecules involved and various effects seen[62]

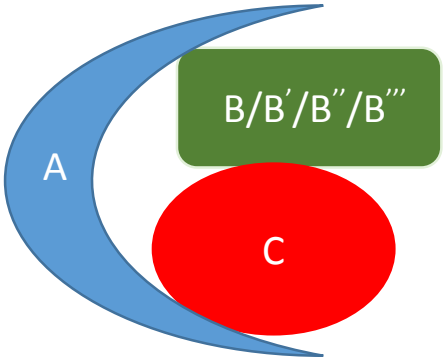

Figure 2. Diagrammatic representation of heterotrimeric PP2A complex

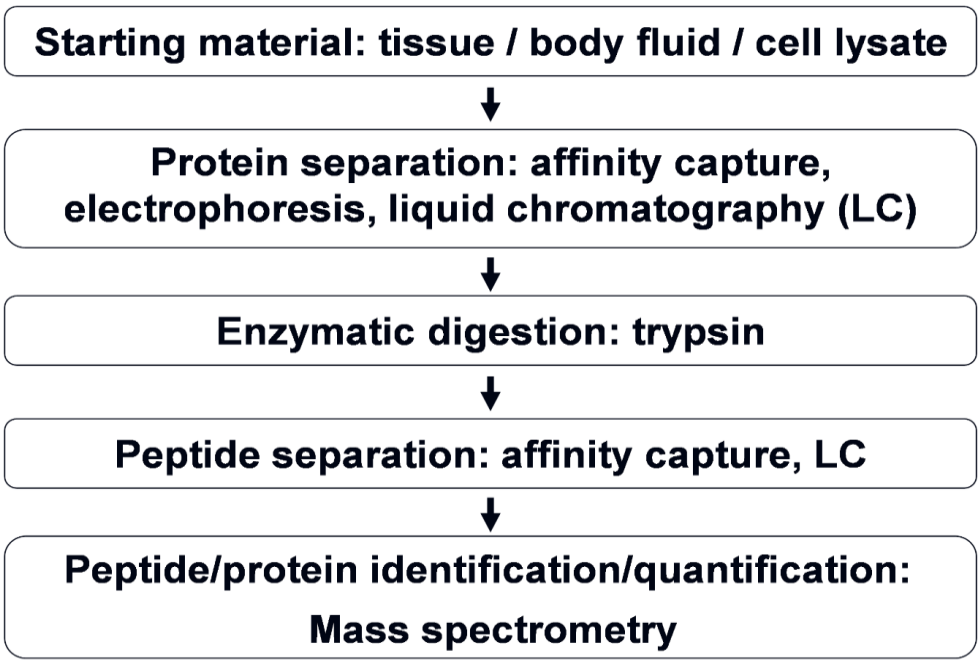

Figure 3. Main steps in mass spectrometry-based proteomics studies

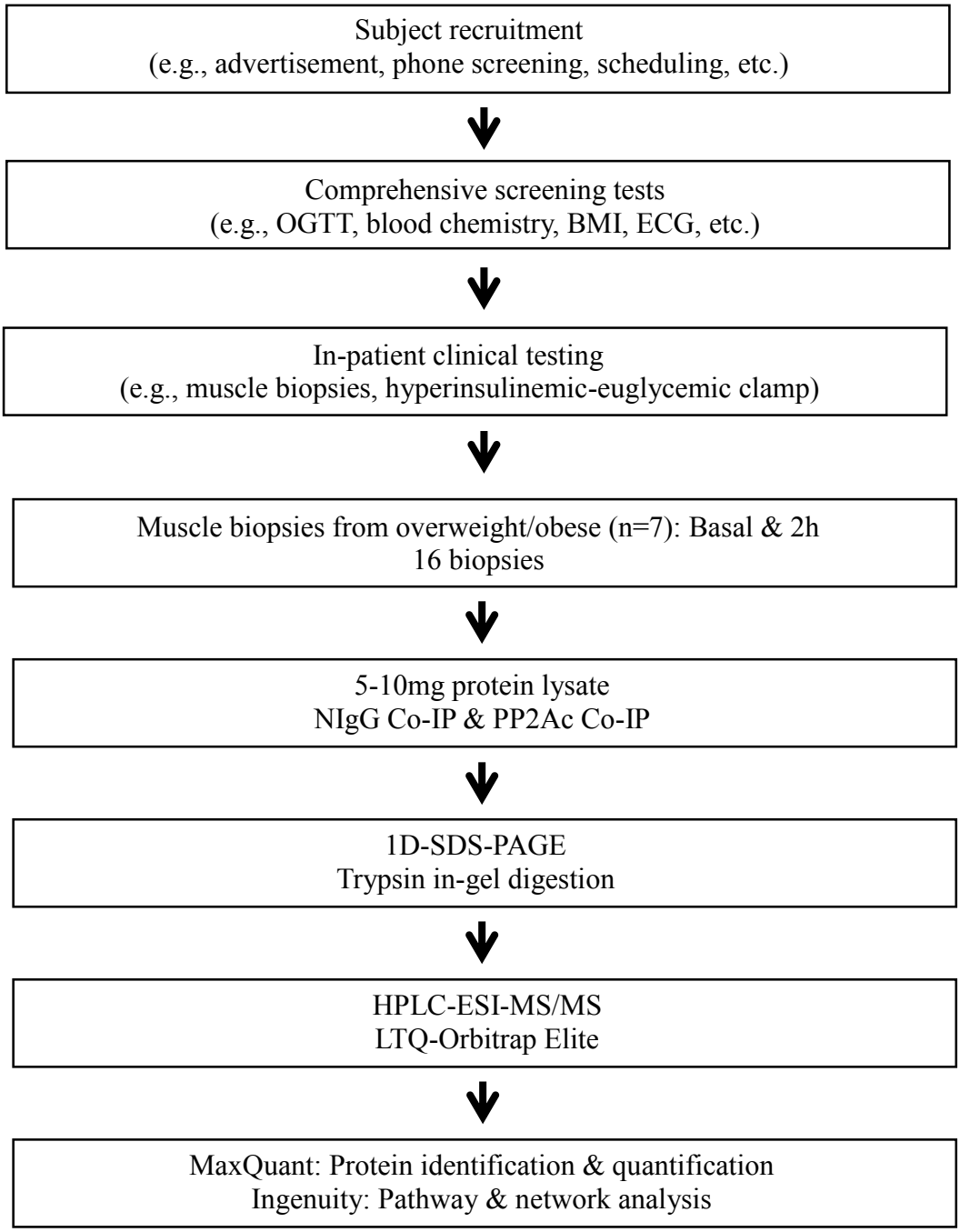

Figure 4. Clinical and proteomics data acquisition and data analysis

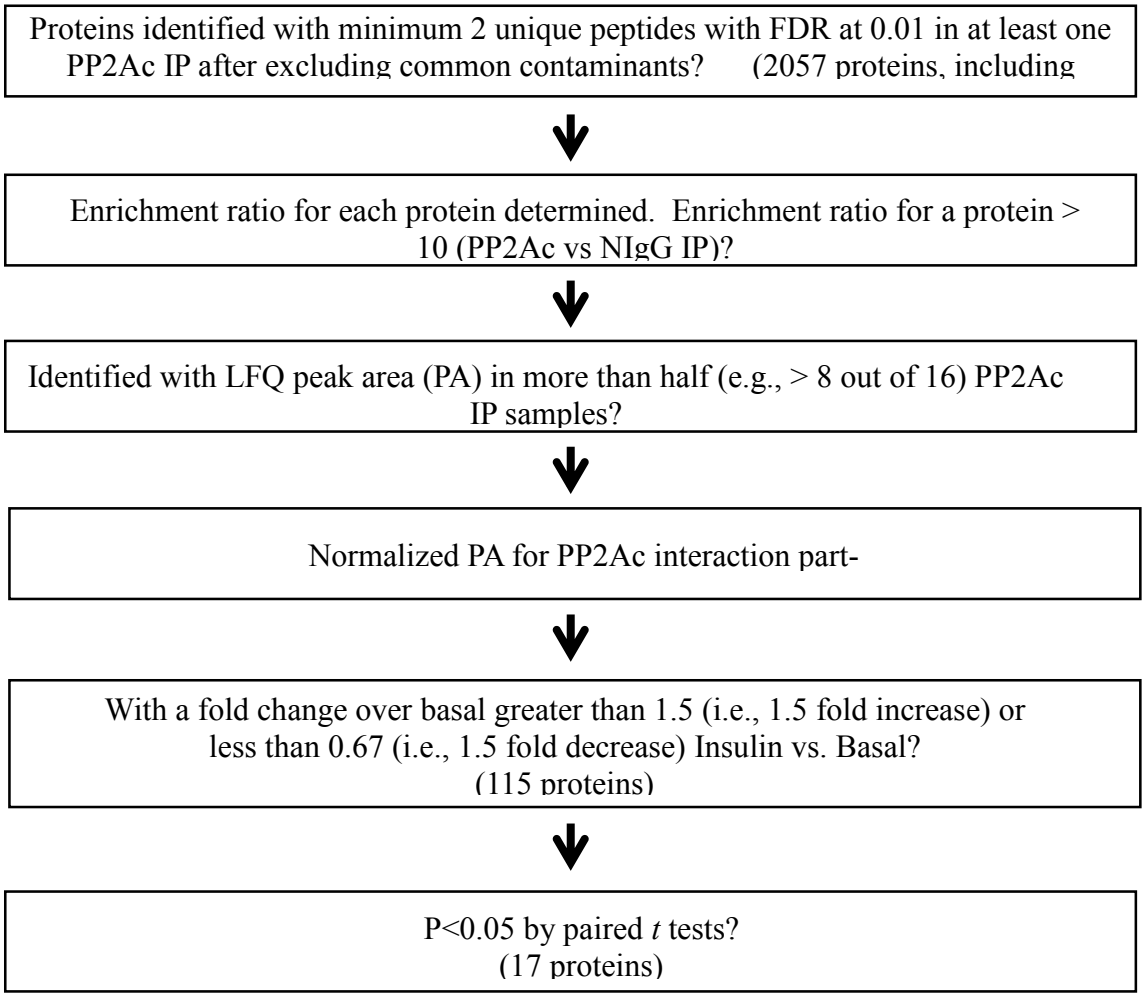

Figure 5. Proteomics data analysis

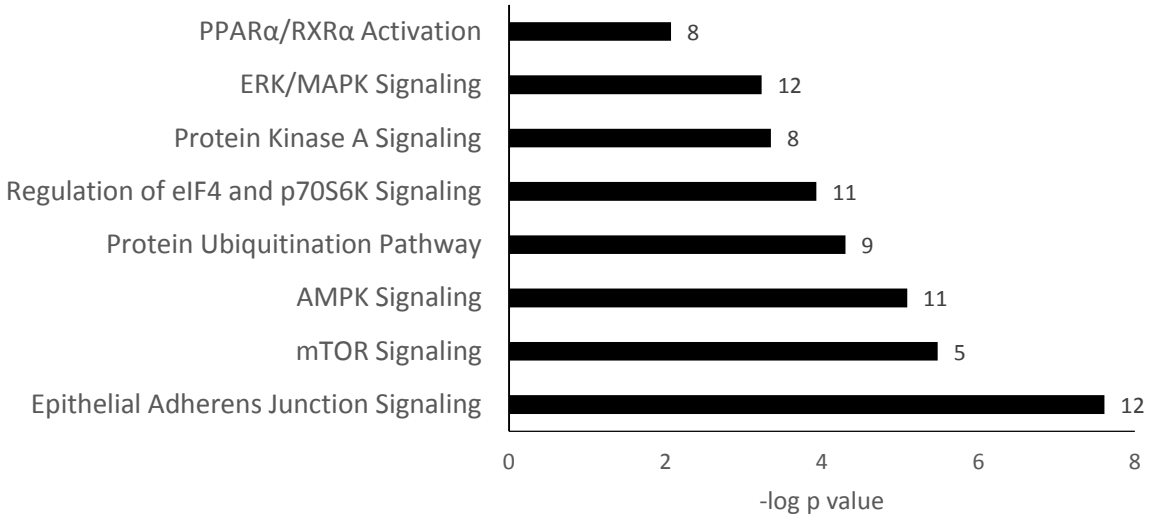

Figure 6. Significantly enriched pathways for the 186 PP2Ac interaction partners and PP2Ac in human skeletal muscle. The number of PP2Ac interaction partners identified in this study in a particular pathway is beside the bar.

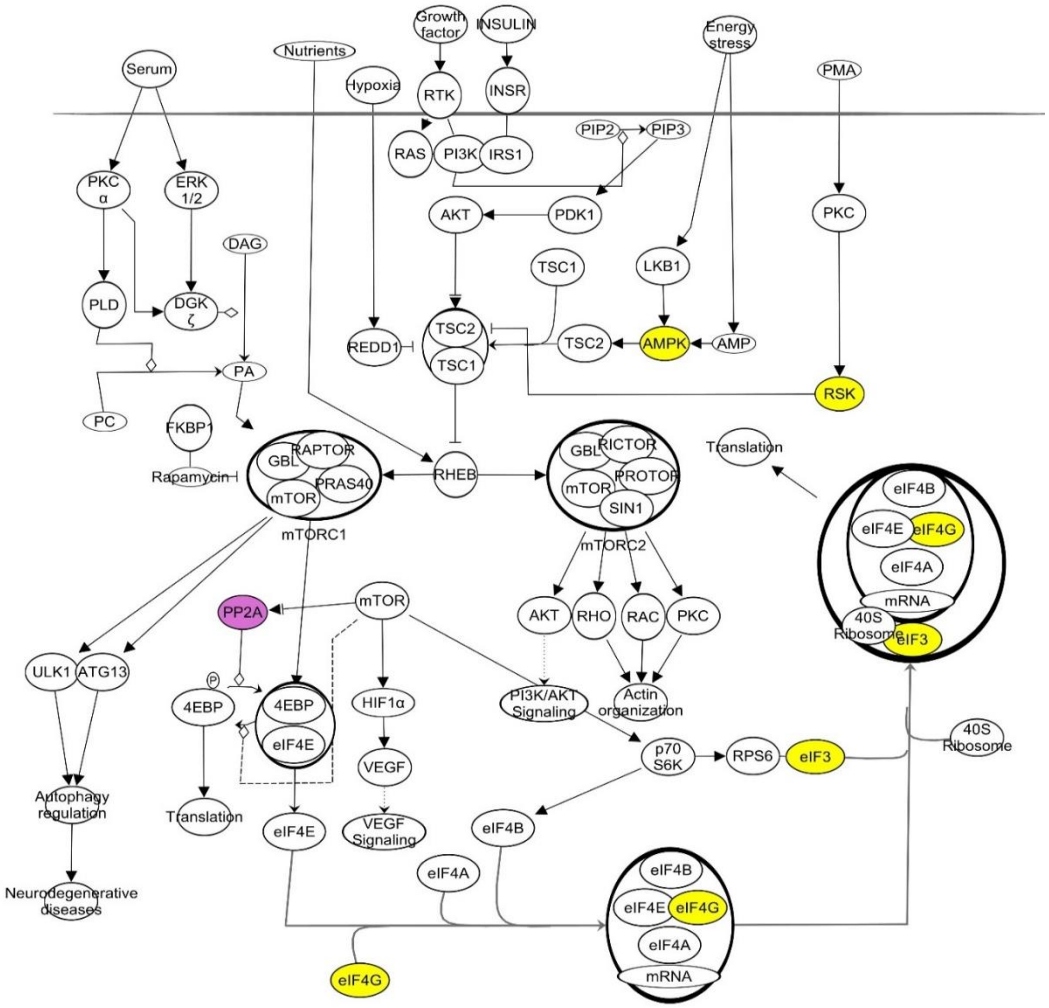

Figure 7. The significantly enriched pathway, mTOR signaling, for the 186 PP2Ac interaction partners and PP2Ac. PP2Ac interaction partners were highlighted in yellow and PP2A was highlighted in purple

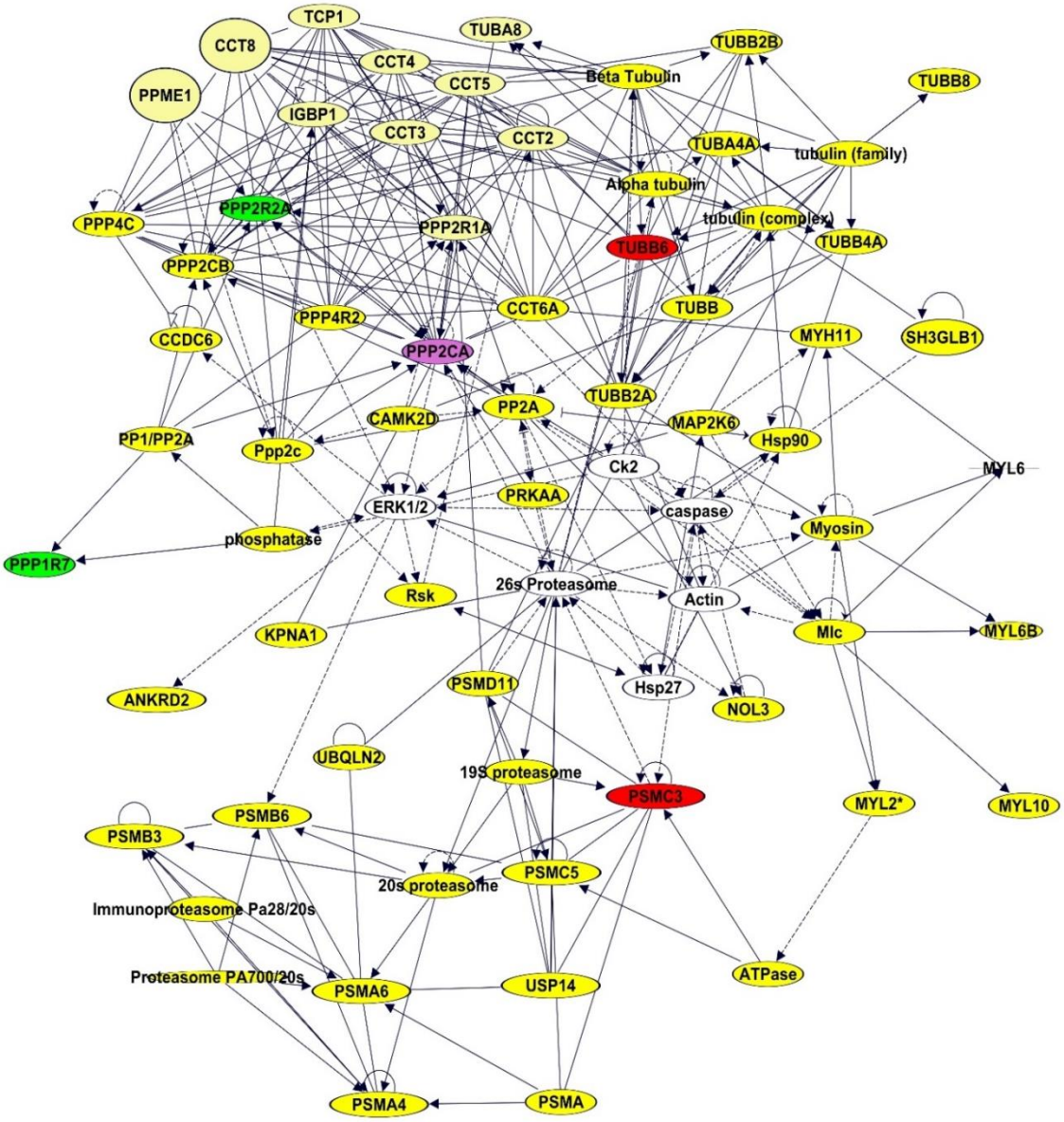

Figure 8. Network pathway obtained from Ingenuity Pathway Analysis Pathway obtained using 70 molecules per network and the one assigned with highest score is taken; shows 45 interaction partners; target protein PP2Ac (in purple) proteins without any change (in yellow), which showed significant decrease (in red) and significant increase (in green) upon insulin stimulation

Table 1 showing the various isoforms of PP2A subunits, their cellular and sub-cellular distribution[8]. HE: high expression

Subunit	Gene	Iso-form	Other name	Normal tissue distribution	Subcellular distribution
Scaffold (A)	PPP2R1A	A	PR65α, PP2A-Aα	Ubiquitously expressed and highly expressed in ovary (oogenesis)	Cytosol
	PPP2R1B	B	PR65β, PP2A-Aβ	Ubiquitously expressed and highly expressed in ovary (oogenesis)	Cytosol
Catalytic (C)	PPP2CA	A	PP2Acα	Brain and heart (HE). Present in skeletal muscle[21]	Cytoplasm and nucleus
	PPP2CB	B	PP2Acβ	Brain and heart (HE)	Cytoplasm and nucleus
Regulatory (B)	PPP2R2A	A	PR55α, PP2ABα	Widely distributed in all tissues	Membranes, cytoplasm, microtubules nucleus. Golgi com-

					plex, endoplasmic reticulum and neurofilaments
	PPP2R2B	B	PR55β, PP2ABβ	Brain and testis (HE)	Cytosol
	PPP2R2C	Γ	PR55γ, PP2ABγ	Brain (SE)	Mainly in Cytoskeletal fraction
	PPP2R2D	Δ	PR55δ, PP2ABδ	Wide spread distribution in tissues, Testis (HE)	Cytosol
Regulatory (B′)	PPP2R5A	A	PR56/61α, PP2AB′α	Cardiac tissues and skeletal muscles[63] (HE)	Cytoplasm
	PPP2R5B	B	PR56/61β, PP2AB′β	Brain (HE)	Cytoplasm
	PPP2R5C	Γ1, 2,3	PR56/61γ, PP2AB′γ	Cardiac tissues and skeletal muscles[64] (HE)	Cytoplasm and nucleus
	PPP2R5D	Δ	PR56/61δ, PP2AB′δ	Primarily exist in brain	Cytoplasm, nucleus, mitochondria, microsomes

	PPP2R5E	E	PR56/61ε, PP2AB′ε	Primarily exist in brain	Cytoplasm
Regulatory (B″)	PPP2R3A	A	PR130, B″α1	Brain (HE), heart, lung, kidney and muscle[65]	Centrosome and Golgi complex
	PPP2R3A	A	PR72, B″α2	Heart (HE) and skeletal muscle[65]	Cytosol and nucleus
	PPP2R3B	B	PR70, PR48, B″β	Placenta	Nucleus
	PPP2R3C	Γ	G5PR, G4-1	During develop-mental process expressed in fetal brain	Nucleus
	PPP2R3D	Δ	PR59, B″δ	Cardiac tissue, kidney and lungs	Nucleus
Regulatory (B‴)	STRN		Striatin, PR110	Brain	Membrane and cyto-plasm
	STRN3		SG2NA	Neurons	Nucleus
	PPP2R4		PTPA, PR53	Widely expressed	Cytosol, nu-cleus

Table 2 showing various inhibitors of PP2A including their sources and specificity to different phosphatases [6].

Inhibitor	**Source**	**Inhibitory potency**
Okadaic acid	*Dinoflagellates*	PP2A ~ PP4 > PP1 ~ PP5 >>> PP2B*
Dinophysistoxin-1	*Dinoflagellates*	PP2A > PP1 >>> PP2B
Microcystins	Cyanobacteria	PP2A ~ PP1 >>> PP2B
Nodularins/Motuporin	Cyanobacteria	PP2A ~ PP1 >>> PP2B
Calyculin A	Isolated from marine sponges	PP2A > PP1 >>> PP2B
Tautomycin	*Streptomyces spiroventricillatus*	PP1 > PP2A >>> PP2B
Cantharidin	Blister beetles	PP2A > PP1 >>> PP2B
Endothall	Synthetic compound	PP2A > PP1 >>> PP2B
Fostriecin	*Streptomyces pulveraceus*subsp. *Fostreus*	PP2A ~ PP4*
TF-23A	Isolated from marine red alga	PP2A
Cytostatin	*Streptomyces sp*. MJ654-NF4	PP2A
I_1^{PP2A}	Cellular inhibitor	PP2A
I_2^{PP2A} (SET, PHAP-II, TAF-1β)	Cellular inhibitor	PP2A

Table 3. Clinical characteristics for the 8 overweight/obese participants in the PP2Ac interaction partner study. Results were shown as mean ± SEM.

Gender *(M/F)*	(4/4)
Age *(years)*	42.0 ± 3.0
BMI *(kg/m^2)*	31.3 ± 01.3
2h OGTT Glucose *(mg/dl)*	115.2 ± 7.8
HBA1c (%)	5.5 ± 0.1
Fasting plasma glucose *(mg/dl)*	92.1 ± 3.5
Fasting plasma insulin *(pmol/l)*	29.0 ± 5.1
M-value *(mg/kg/min)*	5.8 ± 0.6

Table 4. The 186 proteins/ protein groups met the 2 rigorous criteria (See Methods for details) for classification as PP2Ac interaction partners in human skeletal muscle. # indicating previously identified PP2A partners.

ABHD14B	Abhydrolase domain containing 14B
ACAD8	Acyl-coa dehydrogenase family, member 8
ACADM	Acyl-coa dehydrogenase, C-4 to C-12 straight chain
ACADS	Acyl-coa dehydrogenase, C-2 to C-3 short chain
ACADSB	Acyl-coa dehydrogenase, short/branched chain
ACOT9	Acyl-coa thioesterase 9
ACP1	Acid phosphatase 1, soluble
ACTR1B	ARP1 Actin-related protein 1 homolog B, centractin beta (yeast)
ADPRHL1	ADP-ribosylhydrolase like 1
ADSL	Adenylosuccinate lyase
ADSSL1	Adenylosuccinate synthase like 1
ALDH6A1	Aldehyde dehydrogenase 6 family, member A1
ANKRD2	Ankyrin repeat domain 2 (stretch responsive muscle)
APOL2	Apolipoprotein L, 2
ARCN1	Archain 1
ARF4	ADP-ribosylation factor 4
ART3	ADP-ribosyltransferase 3
ASNA1	ArsA Arsenite transporter, ATP-binding, homolog 1 (bacterial)
ATP5A1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle

ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide
ATP6V1B2	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2
ATP6V1E1	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E1
BZW2	Basic leucine zipper and W2 domains 2
CAMK2D	Calcium/calmodulin-dependent protein kinase II delta
CAMK2G	Calcium/calmodulin-dependent protein kinase II gamma
CARM1	Coactivator-associated arginine methyltransferase 1
CCDC6	Coiled-coil domain containing 6
CCT2#	Chaperonin containing TCP1, subunit 2 (beta)
CCT3#	Chaperonin containing TCP1, subunit 3 (gamma)
CCT4#	Chaperonin containing TCP1, subunit 4 (delta)
CCT5#	Chaperonin containing TCP1, subunit 5 (epsilon)
CCT6A#	Chaperonin containing TCP1, subunit 6A (zeta 1)
CCT8#	Chaperonin containing TCP1, subunit 8 (theta)
CD59	CD59 molecule, complement regulatory protein
CDC37	Cell division cycle 37
CECR5	Cat eye syndrome chromosome region, candidate 5
CKM	Creatine kinase, muscle
CKMT2	Creatine kinase, mitochondrial 2 (sarcomeric)
COPA	Coatomer protein complex, subunit alpha
COPS3	COP9 signalosome subunit 3
COQ9	Coenzyme Q9
CRBN	Cereblon

CRYM	Crystallin, mu
CS	Citrate synthase
CUL2	Cullin 2
DARS	Aspartyl-trna synthetase
DCTN2	Dynactin 2 (p50)
DDX3X	DEAD (Asp-Glu-Ala-Asp) box helicase 3, X-linked
DDX3Y	DEAD (Asp-Glu-Ala-Asp) box helicase 3, Y-linked
DDX6	DEAD (Asp-Glu-Ala-Asp) box helicase 6
DHRS7B	Dehydrogenase/reductase (SDR family) member 7B
DIABLO	Diablo, IAP-binding mitochondrial protein
DLD	Dihydrolipoamide dehydrogenase
DLST	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)
DNAJA2	Dnaj (Hsp40) homolog, subfamily A, member 2
DNAJC7	Dnaj (Hsp40) homolog, subfamily C, member 7
DNM2	Dynamin 2
DPYSL3	Dihydropyrimidinase-like 3
EARS2	Glutamyl-trna synthetase 2, mitochondrial
ECHS1	Enoyl coa hydratase, short chain, 1, mitochondrial
EHD2	EH-domain containing 2
EIF2S3	Eukaryotic translation initiation factor 2, subunit 3 gamma, 52kda
EIF3J	Eukaryotic translation initiation factor 3, subunit J
EIF3L	Eukaryotic translation initiation factor 3, subunit L
EIF4G1	Eukaryotic translation initiation factor 4 gamma, 1

ENDOD1	Endonuclease domain containing 1
FAHD1	Fumarylacetoacetate hydrolase domain containing 1
FGB	Fibrinogen beta chain
FH	Fumarate hydratase
FLNB	Filamin b, beta
GCDH	Glutaryl-coa dehydrogenase
GDI2	GDP dissociation inhibitor 2
GFPT1	Glutamine--fructose-6-phosphate transaminase 1
GMPPB	GDP-mannose pyrophosphorylase B
GPD1L	Glycerol-3-phosphate dehydrogenase 1-like
GPT2	Glutamic pyruvate transaminase (alanine aminotransferase) 2
GYG1	Glycogenin 1
HAGH	Hydroxyacylglutathione hydrolase
HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4
HSD17B8	Hydroxysteroid (17-beta) dehydrogenase 8
HSP90AB1	Heat shock protein 90kda alpha (cytosolic), class B member 1
IARS2	Isoleucyl-trna synthetase 2, mitochondrial
IDH3B	Isocitrate dehydrogenase 3 (NAD+) beta
IGBP1#	Immunoglobulin (CD79A) binding protein 1
KLC4	Kinesin light chain 4
KLHL31	Kelch-like family member 31
KLHL40	Kelch-like family member 40
KPNA1	Karyopherin alpha 1 (importin alpha 5)
KPNA3	Karyopherin alpha 3 (importin alpha 4)

KPNA4	Karyopherin alpha 4 (importin alpha 3)
LDHD	Lactate dehydrogenase D
LMCD1	LIM and cysteine-rich domains 1
LYPLAL1	Lysophospholipase-like 1
MAML3	Mastermind-like 3 (Drosophila)
MAOB	Monoamine oxidase B
MAP2K6	Mitogen-activated protein kinase 6
MAPRE2	Microtubule-associated protein, RP/EB family, member 2
MURC	Muscle-related coiled-coil protein
MYH11	Myosin, heavy chain 11, smooth muscle
MYL10	Myosin, light chain 10, regulatory
MYL2	Myosin, light chain 2, regulatory, cardiac, slow
MYL6	Myosin, light chain 6, alkali, smooth muscle and non-muscle
MYL6B	Myosin, light chain 6B, alkali, smooth muscle and non-muscle
NAMPT	Nicotinamide phosphoribosyltransferase
NAP1L4	Nucleosome assembly protein 1-like 4
NDRG2	NDRG family member 2
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)
NEK7	NIMA-related kinase 7
NOL3	Nucleolar protein 3 (apoptosis repressor with CARD domain)
NT5C3A	5'-nucleotidase, cytosolic IIIA
P4HB	Prolyl 4-hydroxylase, beta polypeptide
PACSIN3	Protein kinase C and casein kinase substrate in neurons 3

PCBP2	Poly(rc) binding protein 2
PCBP3	Poly(rc) binding protein 3
PCTP	Phosphatidylcholine transfer protein
PCYOX1	Prenylcysteine oxidase 1
PDHA1	Pyruvate dehydrogenase (lipoamide) alpha 1
PDHX	Pyruvate dehydrogenase complex, component X
PDIA6	Protein disulfide isomerase family A, member 6
PDK2	Pyruvate dehydrogenase kinase, isozyme 2
PGK1	Phosphoglycerate kinase 1
PGM1	Phosphoglucomutase 1
PLIN2	Perilipin 2
PPME1[#]	Protein phosphatase methylesterase 1
PPP1R7	Protein phosphatase 1, regulatory subunit 7
PPP2CA	Protein phosphatase 2, catalytic subunit, alpha isozyme
PPP2CB	Protein phosphatase 2, catalytic subunit, beta isozyme
PPP2R1A[#]	Protein phosphatase 2, regulatory subunit A, alpha
PPP2R2A[#]	Protein phosphatase 2, regulatory subunit B, alpha
PPP3CB	Protein phosphatase 3, catalytic subunit, beta isozyme
PPP4C	Protein phosphatase 4, catalytic subunit
PPP4R2	Protein phosphatase 4, regulatory subunit 2
PRKAA2[#]	Protein kinase, AMP-activated, alpha 2 catalytic subunit
PRKAB2[#]	Protein kinase, AMP-activated, beta 2 non-catalytic subunit
PRKAG1#	Protein kinase, AMP-activated, gamma 1 non-catalytic subunit
PRKAR1A	Protein kinase, cAMP-dependent, regulatory, type I, alpha

PSMA4&6	Proteasome (prosome, macropain) subunit, alpha type, 4 and 6
PSMA6	Proteasome (prosome, macropain) subunit, alpha type, 6
PSMB3;6	Proteasome (prosome, macropain) subunit, beta type, 3;6
PSMC3;5	Proteasome (prosome, macropain) 26S subunit, atpase, 3;5
PSMD11	Proteasome (prosome, macropain) 26S subunit, non-atpase, 11
PTGR1	Prostaglandin reductase 1
RAB14	RAB14, member RAS oncogene family
RAB1B	RAB1B, member RAS oncogene family
RAB6A	RAB6A, member RAS oncogene family
RAB6B	RAB6B, member RAS oncogene family
RDH13	Retinol dehydrogenase 13 (all-trans/9-cis)
REEP5	Receptor accessory protein 5
RNH1	Ribonuclease/angiogenin inhibitor 1
RPL11	Ribosomal protein L11
RPN2[#]	Ribophorin II
RPS6KA3	Ribosomal protein S6 kinase, 90kda, polypeptide 3
RRAGA	Ras-related GTP binding A
RRAGB	Ras-related GTP binding B
RRM2B	Ribonucleotide reductase M2 B (TP53 inducible)
RTN3	Reticulon 3
SCFD1	Sec1 family domain containing 1
SCPEP1	Serine carboxypeptidase 1
SH3GLB1	SH3-domain GRB2-like endophilin B1
SMTNL2	Smoothelin-like 2

SMYD1	SET and MYND domain containing 1
SSBP1	Single-stranded DNA binding protein 1, mitochondrial
STAC3	SH3 and cysteine rich domain 3
STARD7	StAR-related lipid transfer (START) domain containing 7
STAT3	Signal transducer and activator of transcription 3
TBRG4	Transforming growth factor beta regulator 4
TCP1#	T-complex 1
TIMM44	Translocase of inner mitochondrial membrane 44 homolog
TMEM109	Transmembrane protein 109
TMEM143	Transmembrane protein 143
TOM1	Target of myb1 (chicken)
TPM1	Tropomyosin 1 (alpha)
TRIM54	Tripartite motif containing 54
TRIM72	Tripartite motif containing 72, E3 ubiquitin protein ligase
TUBA4A;8[#]	Tubulin, alpha 4a;8
TUBB	Tubulin, beta class I
TUBB2A;2B	Tubulin, beta 2A class iia; 2B class iib
TUBB4A	Tubulin, beta 4A class Iva
TUBB6	Tubulin, beta 6 class V
TUBB8	Tubulin, beta 8 class VIII
UBAC1	UBA domain containing 1
UBQLN2	Ubiquilin 2
UGP2	UDP-glucose pyrophosphorylase 2

UQCRFS1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1
USP14	Ubiquitin specific peptidase 14 (trna-guanine transglycosylase)
VAT1	Vesicle amine transport 1
VPS25	Vacuolar protein sorting 25 homolog (S. Cerevisiae)
VTA1	Vesicle (multivesicular body) trafficking 1
WARS	Tryptophanyl-trna synthetase
ZADH2	Zinc binding alcohol dehydrogenase domain containing 2

Table 5. Significantly enriched pathways for the PP2Ac interaction partners identified in the study revealed by Ingenuity Pathways Analysis.

Ingenuity Canonical Pathways	-log(p-value)	Gene Names	Number of identified PP2Ac interaction partners in the study
Epithelial Adherens Junction Signaling	7.61	TUBB6, TUBA8, MYL2, MYL6, TUBB8, MYL6B, TUBB2A, TUBA4A, TUBB4A, MYH11, TUBB, TUBB2B	12
TCA Cycle II (Eukaryotic)	5.68	CS, DLST, DLD, FH, IDH3B	5
mTOR Signaling	5.48	PPP2CB,PPP2R1A,PRKAB2, PPP2CA, PPP2R2A, PRKAA2, RPS6KA3, EIF3J, EIF4G1, EIF3L	11
AMPK Signaling	5.09	PPP2CB, PPP2R1A, PRKAB2, CKM, PPP2CA, PPP2R2A, PRKAA2, PRKAG1, PRKAR1A	9
Protein Ubiquitination Pathway	4.3	PSMB3+ND6:N6, PSMD11, PSMA6, USP14, HSP90AB1, CUL2, PSMA4, PSMC3, PSMB6, DNAJC7, PSMC5	11

Regulation of eIF4 and p70S6K Signaling	3.93	PPP2CB, PPP2R1A, PPP2CA, PPP2R2A, EIF2S3, EIF3J, EIF4G1, EIF3L	8
Protein Kinase A Signaling	3.35	FLNB, MYL10, CAMK2D, PPP3CB, MYL2, MYL6, PPP1R7, MYL6B, ACP1, PRKAG1, PRKAR1A, CAMK2G	12
PPARα/RXRα Activation	2.07	MAP2K6, PRKAB2, HSP90AB1, PRKAA2, PRKAG1, PRKAR1A	6

Table 6. Interaction partners that showed significant difference (decreased) upon insulin stimulation

Gene name	Protein	Function
ARCN1	Archain1 (Coatomer Protein Complex, Subunit Delta)	Vesicle traffic
ARF4	Adp-ribosylation factor 4	Vesicular trafficking and activators of phospholipase D
COPA	Coatomer protein complex, subunit alpha	Vesicle traffic
GYG1	Glycogenin 2	Primer for glycogen synthesis
PSMC3	Proteasome (prosome, macropain) 26S subunit, atpase, 3	Degradation of proteins
RPL11	Ribosomal protein L11	rRNA maturation and formation of the 60S ribosomal subunits
SCFD1	Sec1 family domain containing 1	Vesicular transport between the endoplasmic reticulum and the Golgi
STAT3	Signal transducer and activator of transcription 3 (acute-phase response factor)	Transcription factor
TUBB6	Tubulin, beta 6 class V	Microtubule protein
VAT1	Vesicle amine transport 1	Vesicle transporter

Table 7. Interaction partners that showed significant difference (increased) upon insulin stimulation:

Gene name	Protein	Function
ADSL	Adenylsuccinate lyase	Catalyzes steps in denovo AMP synthesis
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	Subunit of Mitochondrial protein ATP Synthase; production of ATP
DLD	Dihydrolipoamide dehydrogenase	Mitochondrial enzyme
NT5C3	5'-nucleotidase, cytosolic IIIA	Breakdown of nucleotides
PGM1	Phosphoglucomutase 1	Glucose-1-phosphate to glucose 6 phosphate and Vice versa
PPP1R7	Protein phosphatase 1, regulatory subunit 7	Dephosphorylation of proteins
PPP2R2A	Protein phosphatase 2, regulatory subunit B, alpha	Dephosphorylation of proteins

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ABSTRACT

NOVEL PROTEIN PHOSPHATASE 2A COMPLEXES IN SKELETAL MUSCLE FROM OBESE INSULIN RESISTANT HUMAN SUBJECTS

by

DIVYASRI DAMACHARLA

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Advisor: Dr. Zhengping Yi

Major: Pharmaceutical Sciences (Pharmacology and Toxicology)

Degree: Master of Science

Type 2 Diabetes is a metabolic disorder associated with insulin resistance and consequent high blood glucose levels. Maximum glucose disposal takes place in skeletal muscle and studying skeletal muscle insulin resistance is crucial. Protein Phosphatase 2A (PP2A) is one of the major serine/threonine phosphatases belonging to PhosphoProteinPhosphatase (PPP) family. It constitutes about 80% of all serine/threonine phosphatases. It is regulated by numerous regulatory subunits as well as other substrate molecules and post translational modifications. This alters their localization, activity and also its target molecules. Many evidences show the effect of insulin on PP2Ac and its abnormal regulation in conditions of glucolipotoxicity. Thus, studying PP2Ac interaction partners in respect to type 2 diabetes will give insight into its role in insulin resistance.

Here, we studied interaction partners of PP2Ac in obese insulin resistant human subjects. Two skeletal muscle biopsies, basal and insulin stimulated are obtained from each individual using hyperinsulenemic euglycemic clamp technique. Using ESI-HPLC-MS/MS, we identified 186 interaction partners. Out of which 14 partners were previously identified by other groups which leaves 172 novel partners. This is the largest PP2Ac interaction network found till date. We also identified 17 insulin responsive

PP2Ac partners. Several important PP2Ac interaction partners molecules were identified, for the 1st time, in skeletal muscle from humans. Among them,, some are known to affect PP2Ac activity and others are significantly associated with insulin signaling. Further validation of these partners will help with a better understanding of the role and regulation of PP2Ac in terms of insulin resistance in obese individuals.

AUTOBIOGRAPHICAL STATEMENT

DIVYASRI DAMACHARLA

EDUCATION

2015 Masters in Pharmaceutical Sciences, Wayne State University, Detroit, Michigan, U.S.A.
2012 Bachelors in Pharmacy, Rajiv Gandhi University of Health sciences, India

PROFESSIONAL ASSOCIATIONS

American Society for Mass Spectrometry (ASMS)
American Association for Pharmaceutical Scientists (AAPS)

PRESENTATIONS

Divyasri Damacharla., et al. Identification of Protein Interaction Partners of Protein Phosphatase 2A Catalytic Subunit Using Quantitative Mass Spectrometry, the 62nd American Society for Mass Spectrometry Conference on Mass Spectrometry, June 15-19, 2014, Baltimore Convention Center, Baltimore, MD.