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Proteomic Discovery Of Protein Phosphatase 1 Catalytic Subunit Protein Interaction Partners In Human Skeletal Muscle

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**PROTEOMIC DISCOVERY OF PROTEIN PHOSPHATASE
1 CATALYTIC SUBUNIT PROTEIN INTERACTION
PARTNERS IN HUMAN SKELETAL MUSCLE**

by

Zhao Yang

THESIS

Submitted to the Graduate School

of Wayne State University,

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Advisor

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DEDICATION

To the world beloved

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TABLE OF CONTENTS

LIST OF FIGURES	VI
LIST OF TABLES	VIII
CHAPTER 1 INTRODUCTION	1
1.1 DIABETES MELLITUS AND INSULIN RESISTANCE	1
1.1.1 History of Diabetes	1
1.1.2 Insulin resistance.....	3
1.1.3 Signaling associated with Insulin Sensitivity	4
1.2 PP1C AND PHOSPHORYLATION, AND INSULIN RESISTANCE	5
1.2.1 PI3k-AKT-mTOR Pathway.....	5
1.2.2 Phosphatases: Function, Category and Families.....	7
1.2.3 Introduction of Protein Phosphatase 1 Catalytic Subunit	8
1.3 HYPERINSULINEMIC-EUGLYCEMIC CLAMPS AND SKELETAL MUSCLE	11
1.3.1 Hyperinsulinemic-euglycemic Clamps	11
1.3.2 Tissues response to Insulin	11
1.3.3 Studying skeletal muscle as a promising approach in T2D research	12
1.4 MASS SPECTROMETRY IN DISCOVERY OF PROTEIN-PROTEIN INTERACTIONS	13
CHAPTER 2 RESEARCH DESIGN AND METHODS	15
2.1. MATERIALS.....	15
2.1.1 Antibody	15
2.1.2 Reagents.....	16
2.2. SUBJECTS.....	16
2.3 HYPERINSULINEMIC-EUGLYCEMIC CLAMP WITH MUSCLE BIOPSIES.....	16

2.4 PLASMA INSULIN CONCENTRATION DETERMINATIONS.....	17
2.5 PROTEOMICS SAMPLE PREPARATION AND ANALYSIS.....	17
2.5.1 Muscle biopsy processing	17
2.5.2 Immunoprecipitation, 1D SDS-PAGE, in-gel digestion and mass spectrometry	18
2.5.3 Sample data analysis	19
2.5.4 Statistical analysis	21
2.5.5 Bioinformatics analysis	21
CHAPTER 3 RESULTS	22
CHAPTER 4 DISCUSSION.....	24
4.1 PP1C INTERACTION PARTNERS AS PP1 REGULATORY PROTEINS.....	25
4.2 PP1C INTERACTION PARTNERS INVOLVED IN CYTOSKELETON DYNAMICS	28
4.3 PP1C INTERACTION PARTNERS IN MITOCHONDRIAL FUNCTION.....	30
4.4 INTERACTION PARTNERS ACT IN METABOLISM PATHWAYS.....	31
4.5 INTERACTION PARTNERS RELATE TO PROTEIN SYNTHESIS AND DEGRADATION,	33
4.6 OTHER PP1C INTERACTION PARTNERS.....	37
4.7 INFLUENCE OF MUSCLE FIBER-TYPE COMPOSITION.....	38
4.8 SUMMARY	39
REFERENCES	60
ABSTRACT	80
AUTOBIOGRAPHICAL STATEMENT.....	82

LIST OF FIGURES

Figure 1. The causal relationship and tissue involvement of Type 2 Diabetes development.....	40
Figure 2. Overview of signal molecules involved in insulin signaling pathway....	41
Figure 3. The sequence of three major isoforms of PP1c: PPP1CA isoform 1, PPP1CB, PPP1CC isoform 1.....	42
Figure 4. Structure information of PP1c	43
Figure 5. Interactions between PP1c and its interaction partner, MYPT1 to form a PP1 holoenzyme	45
Figure 6. Regulation patterns of interaction partners on PP1c	46
Figure 7. Summary of glucose metabolism change after Type 2 diabetes among different tissues during euglycemic insulin clamp studies.....	47
Figure 8. Outline of clinical and proteomics data acquisition and analysis. A). General flow chart of clinical and proteomics data acquisition and analysis; B). Detailed illustration of clinical visit 2	48
Figure 9. Detailed Proteomic Analysis workflow.....	50
Figure 10. Network analysis of enriched PP1c interaction partners in human skeletal muscle revealed by proteomics and Ingenuity Pathway Analysis	51

Figure 11. Significantly enriched pathways for the PP1c interaction partners identified in this study revealed by Ingenuity Pathways Analysis. A). Significantly enriched pathways. The total number of identified PP1c interaction partners for a given pathway in this study is denoted beside each bar. B) PP1c interaction partners in EIF2 Signaling.....52

LIST OF TABLES

Table 1. Clinical characteristics participants in the PP1c interaction partner study	54
Table 2. The 46 proteins/ protein groups met the 2 rigorous criteria (See Methods for details) for classification as PP1c interaction partners in human skeletal muscle	55
Table 3. Significantly enriched pathways for the PP1c interaction partners identified in the study revealed by Ingenuity Pathways Analysis.....	59

CHAPTER 1 INTRODUCTION

1.1 Diabetes mellitus and Insulin resistance

Diabetes mellitus, or simply diabetes, is a metabolic disorder that is marked by hyperglycemia, high blood glucose. In 2014, 29.1 million individuals in the United States, which is about 9.3% of the population, have diabetes [1]. Among all cases of diabetes, about 90% to 95% are Type 2 Diabetes (T2D), the diabetes form generally results from defects in insulin action [1]. Furthermore, other related complications of diabetes will afflict patients with their cardinal, ocular, renal, and nervous system dysfunction, mainly resulting from hyperglycemia [2].

1.1.1 History of Diabetes

Unfortunately, though recognition of diabetes has gradually been increasing over 200 years and some progresses have been achieved, there is still no cure for diabetes [3]. One of the possible reasons is that the signaling events related to insulin are quite complicated in both Langerhans islet cells and insulin responsive cells that hundreds of signaling events directly or indirectly involved in the insulin pathway contribute to regulation of insulin level and regulated metabolic function. The relationship of these signaling events with insulin signaling is revealed gradually in several decades, which has been viewed as a remarkable milestone in the history of insulin research. Ironically, discovering something unknown in science is always another step into a new

field, which leads to arising of more mysteries. As a consequence, another aspect for complicity of diabetes lies in the discovering of the mechanism of T2D.

During the early stage in the history of diabetes research, diabetes was considered as a disease due to deficiency of insulin produced by islet cells of Langerhans, which, we know nowadays, is shown in most Type 1 Diabetes (T1D) patients and some Type 2 Diabetes (T2D) patients. Distinction between T1D and T2D has first been made in 1936, based on their different pathogenesis [3].

Type 1 Diabetes usually onsets at very early age of patients, thus it is also named as “juvenile diabetes”, informally. The cause of T1D is hypothesized to be the destruction effect of autoimmune selectively to insulin producing cells, which will lead to further metabolic changes linked to hyperglycemia. Genetic disorder and environment factors may explain most case in T1D [3, 4].

Even though the primary cause of T2D is unclear, continuous research on the metabolic syndrome over several decades has improved awareness of the complex pathogenesis from which the development and the outcome of T2D results. Originally, T2D is thought to develop most often in middle-aged and older population, but in recent years, the upward trend in occurrence of T2D has been shown in young population. Also, the relation between occurrence of T2D and overweight has been affirmed [3, 5]. However, the complexity of T2D attributes most to multi-stage involved in the course of disease development, as well as the interaction between various organs and tissues. Despite the primary factor of the disease is unknown, insulin resistance is consented to be the initiator in early stage which is followed by pancreatic beta cell decompensation [6]. In year 2035,

T2D is expected to agonize more than 530 million people worldwide [7], hence to gain a more thorough understanding of T2D mechanism is considered to be a leading step to conquer the abominable disease.

1.1.2 Insulin resistance

As a major player in T2D development, insulin resistance has been recognized for almost a century and systematically research has been conducted since 1970s [8]. In general, insulin resistance connotes metabolic abnormal state that glucose uptake tissues in an individual are unable to act normally as in healthy population under given quantity of insulin. Individuals with insulin resistance sustain compensatory hyperinsulinemia over hyperglycemia and once the compensation ability of beta cells is overwhelmed by insulin demand, diabetes follows. Also, obesity is considered as a major factor associated with insulin resistance [9]. Thus, researches on the relationship of obesity, insulin resistance and T2D, namely, the “adipo-insulin axis”, have been a hotspot recently [10].

The association of T2D with insulin resistance has been studied for decades, and several lines of evidence have been found [6, 11]:

First, prospective studies illustrate that onset of T2D develops average 1 to 2 decades after the occurrence of insulin resistance. Second, basically insulin resistance is an accordant feature of every T2D patient. Third, insulin resistance works as the best predictor for T2D in the offspring whose parents were T2D patients. Finally, increasing insulin sensitivity is a way to lower the incidence of T2D.

1.1.3 Signaling associated with Insulin Sensitivity

Though insulin sensitivity fluctuates within healthy individuals, and insulin sensitivity occurs normally during puberty, pregnancy, and gaining age [12], the most outstanding factor in diabetes researches is unhealthy lifestyle, and its related disease, obesity. Obesity will further increase the risk for developing T2D and other metabolic syndromes. One of the most distinct characters in obesity state is alteration of releasing Non-esterified fatty acids (NEFAs), hormones and pro-inflammatory cytokines from adipose tissue [12]. These adipose-derived factors will affect other insulin signaling related tissues, including skeletal muscle, liver, and other tissues. The influence will be expressed in functional changes which associate with multiple signaling pathways, which eventually results in the onset of insulin resistance. Two of these pathways, PI3k-AKT and MAPK, are directly activated by insulin receptor-mediated tyrosine phosphorylation of insulin receptor substrates, and other factors involve in inflammatory process, such as TNF- α , IL-6, MCP-1, and IL-8 [13].

The PI3k-AKT pathway, as the most well-known and vital player in insulin signaling, will be provided detailed introduction in the subsequent sections. MAPK pathway activation by insulin signaling functions in gene expression, cell growth and mitogenesis, related to the PI3K-AKT pathway [13]. It has been reported that in the skeletal muscle, T2D and obesity individuals with insulin resistance show abnormally low activity in the PI3K pathway, while relatively normal in the MAPK pathway, compare to healthy individuals [14]. Nevertheless, since MAPK can phosphorylate specific serine sites of IRS1, abnormal activity of MAPK will aggravate insulin resistance when IRS1 has already been impaired

[15]. Additionally, growing evidence has directed to a causative relationship between inflammation and insulin resistance. In obese patients, plasma level of C-reactive protein and inflammatory cytokines shows an incremental change, suggesting that a chronic low-grade inflammation occurs [13]. Other study suggests the inflammation mediators induce insulin resistance through activating JNK and the I κ B kinase- β (IKK- β) pathways [12].

1.2 PP1c and Phosphorylation, and Insulin resistance

Protein phosphorylation is one of the most essential post-transcriptional regulations and being viewed as a principle currency of signaling pathways. Many important cellular processes rely on protein phosphorylation, including insulin signaling. Dysregulation of protein phosphorylation leads to turbulence of cell signaling, and function disorder, such as insulin resistance and T2D. For instance, phosphorylation on serine/threonine sites of IRS1 is considered as a potential source of insulin resistance [16, 17]. As a phosphatase, Protein Phosphatase 1 (PP1) aims to dephosphorylate the protein substrates that have been phosphorylated. Research on PP1 might be a viable way to understand and eventually conquer T2D.

1.2.1 PI3k-AKT-mTOR Pathway

Phosphoinositide 3-kinase (PI3K) pathway has originally been viewed as a component of insulin signaling. However, highly conserved PI3k pathway has also been proved to involve in multiple cellular processes except for insulin

signaling, i.e. cancer. Through later studies, a model of PI3k-AKT-mTOR signal chain has been gradually polishing and finally established [18] (See Fig 2).

As the major trigger of PI3K pathway, insulin molecules relay the signal through insulin receptor to insulin receptor substrates (IRS). As an adaptor protein, activated IRS1 will recruit p85 subunit of class 1A PI3K, which initiates the activation of PI3K. Activated PI3K catalyzes phosphorylation of phosphatidylinositol 4, 5-bisphosphate (PIP₂) to produce phosphatidylinositol 3, 4, 5-trisphosphate (PIP₃). Enhanced PIP₃ level allows binding and activating of PDK1, and the later will phosphorylate and activate PKB/Akt that is also recruit to plasma membrane by PIP₃ [15, 18]. Activated PKB/Akt triggers formation of mTOR/RAPTOR complex which participates in protein synthesis. In addition, PKB/Akt will be a participant in glycogen synthesis through activating GSK3 and in glucose uptake by conducting GLUT4 vesicle translocation mediated by AS160 [18, 19].

As mentioned above, PI3K pathway associates closely with insulin resistance. However, in skeletal muscle, a well-known factor in the PI3K pathway that involves in insulin resistance is serine/ threonine phosphorylation of IRS1. While tyrosine phosphorylation of IRS1 enables binding of downstream proteins in PI3K pathway [20], function of serine/ threonine phosphorylation of IRS is site-specific: either increase or diminish insulin signaling. The dual role of serine/ threonine phosphorylation engenders regulation of different mechanisms, such as tyrosine phosphorylation state of IRS, binding to insulin receptor, subcellular dynamics, and degradation [21]. Though positively regulation of insulin signaling

has been reported, most of serine/ threonine phosphorylation of IRS negatively control insulin signaling [20]. However, in insulin resistance state, usually seen in obesity and T2D patients, serine/ threonine phosphorylation of IRS molecules is abnormally regulated by multiple kinases [22], leading to dysregulation of insulin signaling. Increased in serine/ threonine phosphorylation of IRS is seen as a noticeable tag of insulin resistance in both animal and human studies [15]. Hence, to find a way to dephosphorylate abnormally regulated serine/ threonine sites of IRS might be an accessible avenue to T2D treatment.

1.2.2 Phosphatases: Function, Category and Families

Phosphorylation is one of the most essential and wide-spread post-translational modifications in cells: it rapidly and efficiently alters activity, protein binding and structure of its substrates. Generally, there are two players compete in the area of phosphorylation; protein kinases phosphorylate substrates, while protein phosphatases dephosphorylate substrates. However, not all the amino acids can be phosphorylated; serine, threonine and tyrosine account for most cases in which phosphorylation occurs in mammalian cells.

The classical categorization [23, 24] of phosphatases subdivided them into three basic families: PPP (phosphoprotein phosphatase), PPM (metallo-dependent protein phosphatase), and PTP (protein tyrosine phosphatases) families. The emerging researches [25, 26] replenish more subdivided families on the basis of different structure domains and evolutionary origins, without consensus. Essentially, the PTP members dephosphorylate tyrosine residues only, while the PPP and PPM family comprise of serine / threonine phosphatases;

the PPM family members are Mg²⁺-dependent phosphatases, and the PPP family is a traditional serine / threonine phosphatase family with several well-known members, such as PP1, PP2A, PP2B (calcineurin), PP4, PP5, PP6 and PP7 [27] [28]. Other reviews [25] also include PPEF1 and PPEF2 in this family. These members in PPP family share highly conserved sequence and catalytic motif of their catalytic subunits [26].

Intriguingly, in 518 kinases encoded by human genome, only 90 of them are tyrosine kinases; that is similar with the total number of tyrosine phosphatases [25]. The remaining 428 out of 518 kinases are serine/ threonine kinases [25], which is at least 4 times more than total number of serine/ threonine phosphatases. Over years, much of interest of science researchers focuses on a question raised with the huge divergence between number of serine/ threonine kinases and phosphatases: how do such a limited number of phosphatase offsets phosphorylation by kinases in an organized manner?

1.2.3 Introduction of Protein Phosphatase 1 Catalytic Subunit

As the first serine/ threonine phosphatase has been discovered, studies on Protein Phosphatase 1 (PP1) have never stopped over the past four decades [29]. Many studies have been done on isoforms, structure and functions of PP1. PP1 is not a monomeric enzyme itself; it contains 2 subunits, the Catalytic subunits (PP1c) and the Regulatory subunits (PP1r). Generally speaking, 3 isoforms exist for PP1c: PPP1CA, PPP1CB and PPP1CC. Among each isoforms, highly sequence conservation is observed (Fig 3) —PPP1CA isoform shares 86.7% and 89.8% identity with PPP1CB and PPP1CC, respectively. Alternatively

splicing isoforms exist under each isoforms [28]. Although the 3 isoforms of PP1c encode by different genes, and they show diverse tissue and subcellular distribution, it is safe to regard these isoforms altogether in our research since they share remarkably similar features when they enact their functions in cells [28].

For last decades, it has always been an arduous mission to unravel function motifs of PP1c. In general, two categories of motifs (Fig 4b), binding motifs and catalytic motifs, characterize most essential functions of PP1c. PP1c catalytic core locates in the center of its sequence (residues 41–269 of the α isoform) [30]. Residues associate with catalytic reaction distribute on catalytic core. PP1c engenders catalytic reaction to its substrate simply relies on two divalent metals, usually Iron (Fe^{2+}) and Zinc (Zn^{2+}) in mammalian cells, and Manganese (Mn^{2+}) in bacteria [31]. Metal binding residues on PP1c concentrate in several sites: Asp64, His66, Asp92, Asn124, His173, and His248 [32]. However, to achieve specificity, PP1c also needs to bind other components of PP1 holoenzyme, namely PP1 regulatory subunits (See below). Studies [28] on structure of PP1 holoenzyme unveil that multiple sites contact with regulatory subunits on PP1c. The essential interactions between PP1c and PP1r comprise of 2 groups, polar interaction and hydrophobic interaction, the sites of which disperse in whole sequence. In general, polar interaction occurs on site Asp242, Leu289, Cys291, and Glu287; while hydrophobic interaction appears in site Ile169, Leu243, Asp242, Leu289, Cys291 Phe257, Phe293, and Met290 [33]. On the other hand, researches on direct interaction partners of PP1c manifest the

widespread consensus amino acid sequence, R/K-X(0,1)-V/I-{P}-F/W, where X(0,1) represents none or any amino acid, and {P} stands for any amino acid except for Proline, overlaps through most binding partners [33, 34]. Other docking motif (F-x-x-R/K-x-R/K) has been reported elsewhere [35]. The docking motifs of PP1c could become a predictive clue to discover and validate PP1-interaction partners (PIP).

3D structure of PP1c (Fig 4a) has been illustrated by X-ray crystallography for almost 20 years [36]. Three “grooves”, hydrophobic groove, acidic groove, and C-terminal groove, can be identified from the surface of PP1c (Fig 5), on which PP1c binding sites locate [32, 33].

A protein's role is defined in its interactions with other proteins. PP1 and PP2A are the essential members of PPP family that account for about 90% of phosphatase activity in eukaryotes. Compared with PP2A (contains only 70 holoenzymes), PP1 is reported to form more than 650 holoenzymes. Consequently PP1 is considered to conduct majority of phosphatase activity [31]. Although the number of catalytic subunit of the protein phosphatase is limited and highly evolutionary conserved, a spectrum of interaction partners interacts with each phosphatase, providing specificity (Fig 5). Thus, PP1 regulates its substrates not by increasing gene duplication, but through its outstanding ability to establish interactome. Currently, there are nearly 500 proteins have been predicted to be the PIPs so far. Principally, PIPs either regulate dephosphorylation activity of PP1c, target PP1c to its substrate, or serve as PP1c substrates. In particular, 6 aspects of interaction (Fig 6) can be achieved

through regulation of PIP. Though such a huge number of PIPs has been discovered, there is no relatively large scale PIPs profiling of PP1c (i.e., PP1c interactome) in human skeletal muscle, which is the major aim in this project.

1.3 Hyperinsulinemic-euglycemic Clamps and Skeletal Muscle

1.3.1 Hyperinsulinemic-euglycemic Clamps

As mentioned above, insulin resistance is a predictable signature of T2D and other metabolic diseases. Clinically, to quantify insulin sensitivity, several methods have been used, such as Oral Glucose Tolerance Test (OGTT), Insulin Tolerance Test (ITT), and Glucose Clamps [37]. However, to investigate insulin sensitivity of insulin responsive tissues, e.g. skeletal muscle, the “Gold Standard” is the Hyperinsulinemic-euglycemic Clamp [38], developed by Dr. DeFronzo et al. in 1979 [39].

1.3.2 Tissues response to Insulin

Development of T2D is a combination of actions from multiple tissues. In T2D, insulin resistance in insulin responsive tissues, including Hepatic IR, Adipose Tissue IR, and Skeletal Muscle IR, plays an important role [40]. Liver and adipose tissue are primary targets of insulin. They will react to insulin signal and hence adjust their function in metabolism, such as gluconeogenesis, de novo lipogenesis, and lipolysis. In T2D, several classical effects connect to insulin resistance of liver and adipose tissue, e.g. increased free fatty acid turnover [40] and lipotoxicity [41]. Moreover, liver and adipose tissue also show turbulence of signaling transduction, indirectly affecting other tissues, such as skeletal muscle.

Although brain will not change glucose uptake per se to insulin level, it will process and response to the signal and regulate insulin responsive tissues by signaling transmission [42]. In addition, at early stage of T2D, β -cells are trying to compensate blood glucose increase by producing more insulin. However, when ability to produce insulin is overwhelmed by hyperglycemia, β -cell dysfunction occurs [43].

1.3.3 Studying skeletal muscle as a promising approach in T2D research

As largest energy storage tissues and insulin secretion tissue in human body respectively, liver [40], adipose tissue [41] and Langerhans islet [44] have been well studied. However, it is disappointing to see that studies focusing on another equal important tissue connect with insulin-energy-regulation in human, skeletal muscle have been underestimated. On the contrary to this situation, during T2D, it has long been seen as a fact that the defect in glucose disposal exits mostly in skeletal muscle cells, while liver, adipose tissue, and brain glucose uptake level shows barely significant change between T2D patients and controls (Fig 7). Furthermore, skeletal muscle, as the signal terminal of insulin signaling and other signals associating with insulin resistance, expresses assorted distinct features in aberrant protein-protein interactions during insulin resistance and T2D [45]. However, interaction partners of PP1c in skeletal muscle remain unexplored. Therefore, this project aims to uncover protein interaction partners in skeletal muscle in human.

1.4 Mass Spectrometry in discovery of protein-protein interactions

Multiple methods can be used in studying in PP1c interaction partners. These methods [46] include, but not limited to protein phosphatase assay, yeast two hybrid, PP1 overlay assay, GST pull-downs, co-immunoprecipitation, microcystin-sepharose column, florescence technique, such as FRET [47] and FRAP [48], are widely utilized in identifying PIPs. However, these methods either show low-throughput, low accuracy, or extremely high complicity. As a consequence, proteomic approach combining with mass spectrometry provides an effective solution.

Cells can be simplified as a factory, in which a plenty of workers, such as proteins, DNAs, and RNAs, works on numerous assembly lines (pathways), and finally elicits different products (biological processes). To characterize outcomes of cellular signaling, understanding single component or isolated interaction is much less than enough. Above mentioned traditional methods display limitation in the absence of a “system-wide” [49] view, when they are demanded into the world of proteomics analysis that highly complex network and low abundance of proteins are ubiquitous [50]. And proving a global view is the strength of the mass spectrometry based proteomics approach for protein complexes.

In our lab, we combine HPLC with tandem mass spectrometry (MS/MS). The mass spectrometry instrument in our lab, LTQ-Orbitrap Elite, provides extremely highly mass solution, accuracy along with high sensitivity. In addition, though stable isotope labeling approach increases reproducibility and reduces instrumentation time, the method in this project, label-free approach, protects

samples from chemical contamination originating from labeling agents [51]. Recently, we have developed this label-free approach and have discovered the largest IRS1 interactome in human skeletal muscle and novel abnormalities in IRS1 complexes in T2D [45].

CHAPTER 2 RESEARCH DESIGN AND METHODS

The data in the project primarily derive from two mutually associated aspects, clinical study and proteomics study (Fig 8a). In the clinical study, after the participants were consented, comprehensive screening tests (Visit 1) were performed to ensure eligibility of the participants. Eligible participants were scheduled for the in-patient clinical test (Visit 2) which included hyperinsulinemic-euglycemic clamps with skeletal muscle biopsies (Fig 8b). The muscle biopsy samples collected by clinical study were analyzed by proteomics as follows: biopsy sample homogenization; PP1c co-immunoprecipitation; separating PP1c and its interaction partners by 1D-SDS-PAGE gels; in-gel trypsin digestion and peptide extraction; and HPLC-ESI-MS/MS protein analysis (Fig 8c). Appropriate biological comparison and normal antibody IP are used in order to minimize false positives. Pathway and function analysis were conducted on proteomics data by bioinformatics analysis along with literature search, in order to identify pathways enriched for PP1c interaction partners.

2.1. Materials

2.1.1 Antibody

Two kinds of PP1 α mouse monoclonal antibody (sc-271762 and sc-7482) were purchased from Santa Cruz Biotechnology (Dallas, TX); PP1c β rabbit monoclonal antibody (ab53315) was purchased from Abcam (Cambridge, MA); PP1c β rabbit polyclonal antibody (07-207) was purchased from Upstate/Millipore (Lake Placid, NY).

2.1.2 Reagents

The following suppliers were used: sequencing-grade Modified trypsin (Promega, Madison, WI); protein A sepharose , protein G-Agarose, and iodoacetamide (Sigma, St Louis, MO); C18 ZipTip (Millipore, Billerica, MA); Insulin ELISA Jumbo (AIPCO, Salem, NH).

2.2. Subjects

Nine lean, healthy volunteers were recruited and took part in the study at the C. S. MOTT Clinical Research Center at Wayne State University. The purpose, nature and potential risks of the study were explained thoroughly to all participants, and written consent was obtained before their participation. All participants received a 75 g oral glucose tolerance test on the same day when the consent was obtained and screening tests were conducted to assess glucose tolerance. None of the participants had any significant medical problems (including diabetes), and none engaged in any heavy exercise, and they were instructed to avoid any form of exercise for at least 2 days before the study. The protocol was approved by the Institutional Review Board of Wayne State University.

2.3 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 [45, 52]. On the day of study, the study began at approximately 08:30 hours (time -60 min) after a minimum 10-hour overnight fast. A catheter was placed in an

antecubital vein and maintained throughout the study for infusions of insulin and glucose. A second catheter was placed in a vein in the contra lateral arm, which was covered with a heating pad (60°C) for sampling of arterialized venous blood. Baseline arterialized venous blood samples for determination of plasma glucose and insulin concentrations were drawn. At 09:00 hours (time -30 min) a percutaneous needle biopsy of the vastus lateralis muscle was performed under local anesthesia [53]. Muscle biopsy specimens were immediately 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) a primed, continuous infusion of human regular insulin (Humulin R; Eli Lilly, Indianapolis, IN) was started at a rate of 80 mU m⁻² minute⁻¹, and continued for 120 min. Plasma glucose was collected and measured at 5-min intervals throughout the clamp. Euglycemia was targeted for 90 mg/dl by variable infusion of 20% d-glucose. At 11:30 hours (time 120 minutes), another muscle biopsy was obtained from the contralateral vastus lateralis muscle.

2.4 Plasma insulin concentration determinations.

Plasma insulin concentration was measured by the ALPCO Insulin ELISA Jumbo.

2.5 Proteomics sample preparation and analysis.

2.5.1 Muscle biopsy processing

Approximately 60–80 mg frozen muscle biopsy samples were homogenized in fridge using a Next Advance Bullet Blender (Model BBY5E) in

detergent-containing lysis buffer A (50 mmol/l Hepes, pH 7.6, 150 mmol/l NaCl, 20 mmol/l NaPO₄, 20 mmol/l β-glycerophosphate, 10 mmol/l NaF, 2 mmol/l sodium vanadate, 2 mmol/l EDTA, 1% Triton, 10% glycerol, 2 mmol/l phenylmethylsulfonyl fluoride (PMSF), 1 mmol/l MgCl₂, 1 mmol/l CaCl₂, 10 μg/ml leupeptin, 10 μg/ml aprotinin) at a 100 μl/10 mg ratio. Biopsy specimens were homogenized until no visible muscle remained (~2×5 min). Muscle lysates were then incubated on ice for 15 min followed by centrifugation at 4°C for 20 min at 14× 1,000 rpm. Protein concentrations in the supernatant fractions were determined by the Bradford protein assay (Bio-Rad, Hercules, CA) with BSA as the standard [54].

2.5.2 Immunoprecipitation, 1D SDS-PAGE, in-gel digestion and mass spectrometry

The lysate proteins were precleared with NIgG. A mixture of PP1cα and PP1cβ antibodies coupled to both Protein A Sepharose and Protein G-Agarose beads were used to immunoprecipitate PP1c with its interaction partners from muscle lysate. The immunoprecipitates were spun down and washed three times in 1 ml PBS solution. The remaining PBS solution was removed, the immunoprecipitates were boiled in 15 μl sample loading buffer, and the co-IP proteins were resolved by 1D SDS-PAGE.

The whole SDS-PAGE gel lanes were excised, de-stained twice with 300 μL of 50% acetonitrile (ACN) in 40 mM NH₄HCO₃ and dehydrated with 100% ACN for 15 min. After removal of ACN by aspiration, the gel pieces were dried in a vacuum centrifuge for 30 min. Trypsin in 20 μL of 40 mM NH₄HCO₃ was added

and the samples were maintained at 4 °C for 30 min before the addition of 50µL of 40mM NH₄HCO₃. The digestion was allowed to proceed at 37 °C over-night and was terminated by addition of formic acid (FA). After further incubation, each supernatant was transferred and the extraction procedure was repeated by replenishing new solution with low FA concentration, and the two extracts were combined. The resulting peptide mixtures were purified by C18 ZipTip after sample loading in buffer containing peptides to serve as internal standards [55]. On-line HPLC on a Linear Trap Quadrupole-Orbitrap Elite (LTQ-Orbitrap Elite) was performed as described previously [56] with instrument specific modifications.

2.5.3 Sample data analysis

Using the MaxQuant software, peptides/protein identification and quantification were performed, and peak areas for each protein were obtained by LFQ analysis. Only proteins identified with minimum 2 unique peptides and with false discovery rate (FDR) at 0.01 were considered. To be considered as a bona fide PP1c interaction partner, a protein has to further satisfy following criteria [45]: a). With an enrichment ratio >10 (Fig. 9); b). Identified with LFQ peak area (PA) in more than half of the PP1c IP (i.e. >9 out of 18 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 each protein, Norm:*i*,

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

Then, the average of normalized ratio for each protein in the PP1c co-immunoprecipitates, $\text{Average_Norm: } i_{\text{PP1c}}$, as well as the average of normalized ratio for the same protein in the NIgG co-immunoprecipitates, $\text{Average_Norm: } i_{\text{NIgG}}$, were obtained. Finally, $\text{Average_Norm: } i_{\text{PP1c}}$ was divided by $\text{Average_Norm: } i_{\text{NIgG}}$, to obtain the enrichment ratio for each protein.

$$\text{Enrichment_Ratio: } i = \frac{\text{Average_Norm: } i_{\text{PP1c}}}{\text{Average_Norm: } i_{\text{NIgG}}}$$

Since we used NIgG as a control, the first level of identification will be to search for proteins exclusively detected in the PP1c immunoprecipitates. However, this will result in false negatives. Due to the high sensitivity of our approach, even if a trace amount of a protein was non-specifically absorbed on the NIgG beads, it may be identified with minimum 2 unique peptides with FDR at 0.01. Nonetheless, if this protein is a true component of the PP1c complex, higher peak area will be assigned to this protein in the PP1c sample than in the NIgG sample.

To determine the relative quantities of PP1c interaction partners in lean, healthy subjects, the PA for each protein identified in a specific biopsy was normalized against the PA for PP1c identified in the same biopsy, which results in $\text{Norm: } j$.

$$\text{Norm: } j = \frac{PA_j}{PA_{\text{PP1c}}}$$

The normalization strategy is widely used in proteomics studies involving protein-protein interactions [57], and uses the same concept used in Western blotting. The normalized peak area for each PP1c interaction partner, Norm:j, was compared within the group to assess effects of insulin.

2.5.4 Statistical analysis

Although thousands of proteins were assigned in at least one of 18 biopsies that were detected, a series of filters were used to narrow the number of proteins that were used to assess effects of insulin in lean healthy controls as described above. Statistical significance was assessed using Z-test. Differences were considered statistically significant at $p < 0.05^*$.

2.5.5 Bioinformatics analysis

Pathway analysis on PP1c interaction partners were performed using Ingenuity Pathway Analysis (Ingenuity Systems, Inc., Redwood City, CA), which considers a pathway to be a set of genes. IPA software package is widely used and contain biological and chemical interactions and functional annotations created by manual collection of the scientific literature [58]. A pathway was considered as significantly enriched if both the false discovery rate (FDR) for the pathway was less than 0.01 and the pathway included at least 4 of the identified PP1c partners.

CHAPTER 3 RESULTS

Clinical information of participants in the project is shown in Table 1. Criteria indicating the state of diabetes are shown in the table, namely 2h OGTT Glucose level, HBA1c percentage, and Fasting plasma glucose level, manifesting the values within healthy, non-diabetes range.

PP1 α and PP1 β were detected in PP1c immunoprecipitates from all 18 biopsies used for the study, but were not detected at all in the NlgG immunoprecipitates. In total, 46 proteins met the criteria for classification as PP1c interaction partners (Table 2). Note that PP1c interaction partners listed in Table 2 may involve both direct interaction partners, and indirect partners through other proteins that interact with PP1C directly. Among these 46 PP1c interaction partners, 31 partners were previously unreported in any species. Among the 15 proteins were previously reported as PP1c interaction partners, only 3 were reported in human skeletal muscle (Table 2). IPA pathway analysis on the 46 PP1c interaction partners manifested that multiple pathways are significantly enriched, such as pathways related to mitochondrial function, insulin signaling, protein synthesis and degradation, and cytoskeleton dynamics (Fig 10A and Table 3). These results imply that PP1c participates in these biological processes or that PP1c is a downstream target regulated by these pathways. A significantly enriched interaction network of PP1c (EIF2 Signaling) is shown in Fig 10B. These novel PP1c interaction partners in humans may be a reference of the various roles that PP1c plays in physiological normal conditions in skeletal

muscle and other tissues/organs. It also provides a control for PP1c interaction partner researches in insulin resistance and T2D.

Among 46 interaction partners, 8 showed significant change in their interaction to PP1c between basal (without insulin-infusion *in vivo* in humans) and insulin (after 2 hour insulin-infusion *in vivo* in humans) biopsies; 6 of them significantly increased, and other 2 decreased. In addition, 23 interaction partners show 1.3 fold, non-significant change after insulin stimulation (among them, only one had decreased association with PP1c). Please see Table 2 for details.

CHAPTER 4 DISCUSSION

During the past decade, genomics and transcriptomics studies have revealed valuable information regarding mechanisms underlying insulin resistance and T2D. However, the abundance of proteins and protein-protein interactions may not be assessed directly using genomics and transcriptomics [59]. HPLC-ESI-MS/MS based proteomics has emerged as a prevailing means for investigating protein-protein interaction networks in insulin resistance. PP1c interaction partners are large in number and widespread in various tissues and cell types. However, most studies on PP1c protein-protein interactions have been performed in cell culture or animal models. No PP1c interactome in human skeletal muscle tissue has been reported yet. The present project determined interacting partners of PP1c in muscle biopsies of lean healthy participants using the proteomics approach recently developed in our group [45]. The proteomics approach includes NiGg immunoprecipitation as a negative control to determine non-specific binding, multiple biological comparisons to improve confidence, and bioinformatics analysis to identify significantly enriched pathways. The strategy detects endogenous protein complexes, without using labeling or protein overexpression/ tags [45], may be applicable to other protein complexes in cells, animal models, and in human tissue samples [45]. Using this approach, we have identified 46 PP1c interaction partners in multiple functional pathways in small skeletal muscle biopsies from human participants. These interaction partners are discussed below.

4.1 PP1c Interaction Partners as PP1 regulatory proteins

PPP1R2/PPP1R2P3 protein group

Protein phosphatase inhibitor 2 (PPP1R2), used to be called as I-2, was one of the first PP1c inhibitors discovered. In contrast to the other PP1c inhibitors, PPP1R2 inhibits PP1c by competitive inhibition. Unlike PPP1R1 and DARPP-32, classical PP1c RVXF binding motif is not seen in sequence of PPP1R2, which suggests that PPP1R2 interacts with PP1c in a different way with PPP1R1 and DARPP-32 [60]. Despite extensive studies, the physiological role and mechanism of PPP1R2 remain elusive. Interestingly, PPP1R2 has been report to induce centrosome separation [61], and fluctuation of PPP1R2 protein and mRNA levels during the cell cycle is observed [62]. PPP1R2 was first discovered in 1976 in rabbit skeletal muscle [63]. Though it is ubiquitous expressed, no research has shown its function and interaction partners in human skeletal muscle.

Protein phosphatase 1, regulatory (inhibitor) subunit 2 pseudogene 3 (PPP1R2P3) was previously thought to be a pseudogene. However, it has been recently identified at the protein level [64]. It has similar function with PPP1R2 and shares 95% sequence identity with PPP1R2 [64]. The peptides identified by HPLC-ESI-MS/MS were shared between PPP1R2/PPP1R2P3.

Protein phosphatase 1 regulatory subunit 3A and 3B (PPP1R3A, PPP1R3B)

PPP1R3A and PPP1R3B are glycogen-associated regulatory subunits of PP1c, PP1-GTSs, which direct PP1c to the glycogen particles. Several other members exist in PP1-GTS family. However, members in this family are not

characterized by their low sequence identity, but by glycogen-binding domain [65]. Both PPP1R3A [66] and PPP1R3B [67] have been reported to interact with PP1c in human skeletal muscle. PPP1R3A specific expressed in myocytes, while PPP1R3B can express both in muscle and in liver [65].

Protein phosphatase 1 regulatory subunit 7 (PPP1R7).

PPP1R7 is homolog of *Schizosaccharomyces pombe* gene *sds22* in humans. It is expressed in a variety of tissues in humans, including skeletal muscle [68]. In *S. pombe* studies, it is clear that *sds22* regulates chromosome segregation during mitosis [69]. PPP1R7 also involves in regulation of cell shape and myosin phosphorylation state [70].

Nuclear inhibitor of protein phosphatase 1 (PPP1R8)

PPP1R8, also commonly known as NIPP1, is one of the evolutionarily oldest PP1 regulators. PP1c interaction complexes with PPP1R8 possess more than one-third of the nuclear pool of PP1 holoenzyme [71]. PPP1R8 was originally identified as a PP1 inhibitor because binding of NIPP1 inhibits the dephosphorylation of canonical PP1 substrates, including glycogen phosphorylase a [72]. Although mechanism of functions of PP1-PPP1R8 complex remains uncertain, it is reported that the complex binds RNA to exert endoribonuclease activity [73]. Other functions that associate to the complex include transcription, pre-mRNA splicing, cell-cycle progression, and/or chromatin remodeling [72]. PP1-PPP1R8 complex targets a number of substrates, including the pre-mRNA-splicing factor SF3B1, a component of the

U2 snRNP that recruits the complex to the spliceosome [74]. Intriguingly, SF3B1 has also been found as an interaction partner in this research (Table 2).

Protein phosphatase 1 regulatory subunit 11 (PPP1R11),

PPP1R11, also known as Inhibitor-3 of PP1c, is a small protein that is conserved evolutionarily. PPP1R11 is localized to the nucleoli and centrosomes by regulation of different PP1c isoforms. A significant portion of the cellular pools of PP1 α and PP1 γ 1 that are associated with PPP1R11 is reported, suggesting that PPP1R11 may modulate cellular pool distribution and subcellular localization of these isoforms [75]. Though still being unclear, cellular functions of PPP1R11 are likely to be associated with nuclear regulation of PP1 and with the regulation of cell division [76]. PPP1R11 may also have a role in apoptosis [77]. In mammalian cells, PPP1R11 is hetero-terpolymerized with PP1c and PPP1R7, a PP1 regulatory subunit that is involved in mitosis and chromosome segregation processes [78]. Interestingly, PPP1R7 has also been found as an interaction partner in this research (Table 2).

Protein phosphatase 1 regulatory subunit 12B (PPP1R12B)

PPP1R12B, also known as MYPT2, forms complex with PP1c β . PPP1R12B is expressed preferentially in heart, brain and skeletal muscle, while another member in MYPT family, MYPT1 (PPP1R12A) expresses mainly in other cell types such as smooth muscles [79]. PPP1R12B is involved in muscle contraction, cardiac torsion, and organization of sarcomere [80]. Interestingly, PPP1R12B, as well as PPP1R12A, has been found to associate with insulin signaling. Pham et al. [81] reported previously that PPP1R12B phosphorylation is

responsive to insulin stimulation, indicating PPP1R12B may be involved in insulin signaling. Geetha et al. [82] reported that in L6 cells, PPP1R12A/PP1c β may involve in insulin signaling depending on Akt and mTOR/raptor activation.

Phosphatase and actin regulator 4 (PHACTR4)

PHACTR4 is a PP1 regulatory protein, mediating both PP1 and actin binding. Protein members in PHACTR family have been implicated in many distinct biological processes depending on actin cytoskeleton, including angiogenesis, cell spreading, migration and axon elongation. PHACTR4 is reported to interact with PP1c by yeast two hybrid [83]. The PHACTR4 mutation cannot interact with PP1, resulting in cell cycle regulation abnormality. Actin binding to PHACTR4 competes with PP1 binding and consequently the PP1c phosphatase activity is determined by the ratio of monomeric and polymeric actin in cells [84].

4.2 PP1c Interaction Partners involved in cytoskeleton dynamics

ACTB/ACTG1 protein group

Actin β and γ 1 are actins. Actin is one of the well- studied proteins in cells. There are 6 actin isoforms: 4 muscle actins, 2 cytoplasmic actins. Beta- and Gamma actin are cytoplasmic actins. Beta- and Gamma actin are nearly identical (differ by only 4 amino acids), and the peptides identified by HPLC-ESI-MS/MS were shared by these two proteins. Generally, actin plays an important role in cytoskeletal structure, cell mobility and cell morphology [85] [86]. However, distinct co-localization patterns and function occurs among isoforms. To be specific, beta-actin localizes near the leading edges in several cell types, and it

may elicit essential function in neuronal development. Gamma actin can be detected in filament structure encircling mitochondria. Unlike beta isoform, gamma actin distributes evenly in fibroblasts. ACTB knockout is lethal mutation, while ACTG1 knockout is not [85] [86]. Actin beta and gamma1 is reported to interact with PP1c in cell model [87] and in rat tissues [88]. ACTB mRNA is regulated by insulin in human skeletal muscle [89].

Prothrombin (F2)

F2 is one of the coagulation factors that functionally relates to vascular endothelial growth factors [90]. It has also been reported that F2 stimulates actin contraction in LM8 cell lines [91].

LIM and cysteine-rich domains protein 1(LIMCH1)

LIMCH1 contains both LIM and Calponin homology domains, so that it may function as cytoskeletal organization [92] and/or actin binding [93].

Prolactin-inducible protein (PIP)

PIP is small in size and plays multiple important functions. It has ability to bind potentially with CD4-T cell receptor, immunoglobulin G (IgG), actin, zinc α 2-glycoprotein (ZAG), fibronectin and enamel pellicle, revealing its importance in biological functions [94].

Erythrocyte band 7 integral membrane protein (STOM),

STOM, also known as stomatin, shows co-localization with actin microfilaments in epithelial cells [95]. STOM is located in protruding structures in plasma membrane and it forms homo-oligomers in the human epithelial cell, suggesting that this protein participates in the cortical morphogenesis of the cells

[96]. However, other roles of STOM in cells are still in opaque, e.g. overexpression of STOM negatively affects activity of GLUT-1 glucose transporter [97].

TRIO and F-actin-binding protein (TRIOBP)

TRIOBP has three isoforms, but only TRIOBP-1 is ubiquitously expressed. Cellular structure functions by directly binding F-actin as well as associating with the trio rho guanine nucleotide exchange factor to regulate actin filament organization [98] and adherens junctions [99] in cells.

4.3 PP1c Interaction Partners in mitochondrial function

Isobutyryl-CoA dehydrogenase, mitochondrial (ACAD8)

ACAD8 is one of the flavoproteins. The encoded protein is a mitochondrial enzyme that functions in catabolism of the branched-chain amino acid valine [100]; Defects in this gene are the cause of isobutyryl-CoA dehydrogenase deficiency [101]. ACAD8 is reported to interact with PP1c by yeast two hybrid [83].

Estradiol 17-beta-dehydrogenase 8 (HSD17B8)

HSD17B8 namely, 17 beta-HSD8, was previously classified as a steroid-metabolizing enzyme [102], but recent data suggest that HSD17B8 is primarily involved in mitochondrial fatty acid synthesis [103].

Metaxin 2 (MTX2)

MTX2 is bound to the mitochondrial outer membrane at the cytosolic face by its interaction with membrane-bound metaxin 1. This complex may play a role in protein importation into mammalian mitochondria [104].

Pentatricopeptide repeat-containing protein 1 (PTCD1)

PTCD1 is a mitochondrial matrix protein that associates with leucine tRNAs and precursor RNAs that contain leucine tRNAs [105].

Mitochondrial import inner membrane translocase subunit TIM50 (TIMM50)

TIMM50 encodes Tim50 protein that is a Subunit belonging to TIM23 Complex. The TIM23 complex links protein translocation across the Outer and Inner membranes of mitochondria [106]. The existence of a CTD-like phosphatase domain in human Tim50 suggests that Tim50 might exert a serine/threonine phosphatase activity in vitro [107]. Intriguingly, this phosphatase activity is not conserved evolutionarily. The phosphatase activity shows in TIM50 homolog in *Trypanosoma brucei*, a parasitic protozoan; while it is not in Fungal Tim50 [108].

Uveal autoantigen with coiled-coil domains and ankyrin repeats (UACA)

UACA is an Autoantigen that is regulated by insulin in human skeletal muscle [89]. Autoantigen may be attacked by autoimmunity which leads to Vogt-Koyanagi-Harada disease [109] or Graves' disease [110]. The mouse ortholog of UACA may play some roles in cardiac muscle development, cytoplasm, especially around the nuclear membrane and mediates apoptosis [111].

4.4 Interaction Partners act in metabolism pathways

Very long-chain specific acyl-CoA dehydrogenase, mitochondrial (ACADVL)

ACADVL acts on its substrate on 14-24 carbon chain length with optimum activity for palmitoyl-CoA (C16-CoA) in β -oxidation in skeletal muscle [112].

Caveolin-1(CAV1)

CAV1 is an integral membrane protein with multiple functions. The preferred location for caveolin-1 is the caveola at the cell surface. It involves in a number of cellular functions. The most common function for caveolin-1 is to be a lipid transporter. Cholesterol is required for lipid transportation of caveolin-1, and caveolin-1 also binds to long chain unsaturated fatty acids to facilitate its transportation [113]. Caveolin-1 not only transports lipids, but also conducts membrane traffic of other components [114]. Moreover, though most researchers believe caveolin-1 to be a scaffold protein, some reports show activation of EGF pathway leads to down-regulation of caveolin-1, which may aggravate tumor invasion ability [115]. Finally, caveolin-1 acts as an activator of insulin signaling [116], and caveolin-1 deficient mice have been shown to be insulin resistance [117]. Caveolin-1 has been shown to interact with PP1 in prostate cancer cell models [118].

3-methyl-2-oxobutanoate dehydrogenase kinase, mitochondrial (BCKDK)

The mammalian mitochondrial branched-chain α -keto acid dehydrogenase (BCKD)1 complex conducts catalysis of the oxidative decarboxylation structure of branched-chain α -keto acids to bring about branched-chain acyl-CoAs formation. The reaction products indirectly associate with the Krebs cycle or linked to lipid biosynthesis. BCKDK is the kinase that regulate this process [119].

Cystatin-B (CSTB)

CSTB has been suggested to counteract inappropriate proteolysis of the cell due to cathepsins that leak out of the lysosomes, but CSTB may also interact with other cellular proteins [120].

PDZ domain-containing protein GIPC1 (GIPC1)

GIPC1 is a G-couple protein. In L6 myoblast cells, GLUT1/GIPC1 interaction increases with enhanced GLUT1 activity, which may participate in glucose uptake regulation [121].

4.5 Interaction Partners relate to Protein Synthesis and Degradation,

Splicing factor 3B subunit 1 (SF3B1)

SF3B1 also known as Sap155, belongs to U2 spliceosomal RNA (snRNP) that is a component of spliceosome. Spliceosome processes precursor mRNAs into mature mRNA by splicing intron. Posttranslational protein modification, especially phosphorylation state, is critical for splicing dynamics, e.g. SF3B1 is hyperphosphorylated before and dephosphorylated after step 1 of splicing [122]. It has also been reported that in HTO cells, dephosphorylation activity of SF3B1 is conducted by Protein Phosphatase 1, regulated by Nuclear Inhibitor of Protein Phosphatase-1 (NIPP1, also known as PPP1R8) [123]. This is considered as an evidence that PP1 involves in regulation of transcription and protein synthesis.

Heterogeneous nuclear ribonucleoprotein Q (SYNCRIP)

As mentioned above, pre-mRNA converts into mature mRNA by spliceosomes. However, another group of proteins, Heterogeneous nuclear ribonucleoproteins (hnRNPs), involves before and after pre-mRNA processing. hnRNPs prevent pre-mRNA forming a short secondary structure, leading to better accessibility for interactions. In addition, hnRNPs function as mRNA transporter that assists mature mRNA to transport out to cytoplasm [124]. Research has shown that the cytoplasmic RNA-binding protein, SYNCRIP, was highly homologous to heterogeneous nuclear ribonucleoprotein R (hnRNP R). SYNCRIP binds to RNAs with preference to poly-(A) RNA. Nevertheless, distribution of SYNCRIP is predominantly in the cytoplasm, while the nuclear localization is shown in hnRNP R [125]. Like hnRNPs, SYNCRIP function as a stabilizer of mRNA [126]. SYNCRIP has been shown to interact with PP1 in cell models [87].

Transformer-2 protein homolog beta (TRA2B)

The serine- and arginine-rich protein (SR protein) of RNA binding proteins plays an important role in both constitutive and alternative pre-mRNA splicing. It is known to be a molecule that involves in both protein–protein and protein–RNA interactions. These interactions are important for RNA metabolism. As a member of SR protein family, TRA2B structurally associates with the classical SR proteins and functionally involves in regulating alternative splicing pathways. Mammalian TRA2B has been shown to influence tissue specific functions by appropriate

alternative splicing [127]. An in vitro study has shown the interaction between TRA2B and PP1 [128].

39S ribosomal protein L49 (MRPL49)

MRPL49 is a component in human mitochondrial ribosomes [129].

DNA-directed RNA polymerase II subunit RPB3 (POLR2C)

POLR2C is a subunit of RNA polymerase II. In eukaryotic cells, transcription of protein coding genes is accomplished by RNA polymerase II, associating with a number of cofactors. These cofactors control the selectivity and efficiency of transcription initiation, elongation and finally termination [130].

DNA-directed RNA polymerase II subunit RPB1 (POLR2A)

POLR2A is another component of RNA polymerases that functions to remove the phosphates from the RNA polymerase II carboxyl-terminal domain. RNA polymerases are recruited to target gene, forms a transcription complex, and initiates transcription process. In eukaryotes, three types of RNA polymerase exist, each of which targets different RNAs. RNA polymerase II participates in transcriptions of all protein-coding genes, producing mRNA [124]. Insulin stimulation on skeletal muscle increases in the muscle transcript levels [131], which putatively increases RNA polymerases II activity. Furthermore, RNA polymerases II binds to PPAR γ , a transcriptional activator in adipocytes, when it transcribes genes involved in lipid synthesis, lipids storage, cell growth, insulin signaling, and adipokine production [132]. This protein has been found in vitro to interact with PP1c [133].

Protein quaking (QKI)

QKI is an RNA binding protein that regulates embryogenesis, blood vessel development, glial cell fate determination, and apoptosis [134].

RNA-binding protein Raly (RALY)

RALY is a member of the heterogeneous nuclear ribonucleoproteins, a family of RNA-binding proteins generally involved in many processes of mRNA metabolism [135].

Ribosomal proteins: RPL14, RPL18A, RPL3L, RPS11, RPS25, and RPS9

The ribosome is a large and complex molecular machine that serves as the primary site of biological protein synthesis [136]. Ribosomes are large ribonucleoprotein complexes that provide an accurate structure for mRNA translation and protein synthesis [137]. The eukaryotic ribosome includes four ribosomal RNA (rRNA), and large numbers of ribosomal proteins (RP) [138]. This complex exhibits a very huge molecular mass and a sedimentation coefficient of 80S. The ribosome is composed of two major subunits; a small subunit involved in decoding of the mRNA and a large subunit that embraces the peptidyl transferase center which is buried in the rRNA. In eukaryotes, the 40S small subunit is composed of only one 18S rRNA, whereas the 60S large subunit contains three rRNA (5S, 5.8S and 28S) [137]. Among the list of ribosomal proteins in this study, RPS11, RPS25, and RPS9 belongs to 40s subunit while RPL14, RPL18A, and RPL3L belongs to 60s subunit [137, 139].

Double-stranded RNA-binding protein Staufen homolog 2 (STAU2)

STAU2 is regulated by insulin in human skeletal muscle [89]. Function of STAU2 includes binding of double-stranded RNA, transport of RNAs in neuron, degradation of specific mRNAs contains STAU-binding site 1 [140].

Transcription elongation factor B polypeptide 2 (TCEB2)

TCEB2 is a component of E3 ubiquitin ligase, conduct ubiquitination [141]. It serves a chaperone-like function, facilitating assembly and boosting stability of the Elongin (SIII), a complex activates translational elongation process by mammalian RNA polymerase II. The elongation process is initiated by minimizing transient pausing of the polymerase within transcription units [142].

Probable tRNA pseudouridine synthase 2 (TRUB2),

TRUB2 also known as TruB, is an RNA pseudouridine synthase which catalyzes pseudouridine formation in tRNA. This posttranscriptional modification is evolutionary conserved [143]. Another member in TRUB family, TRUB1, is widely expressed in various human tissues (especially heart, skeletal muscle and liver). TRUB2 gene was also found in human genome, however, much of the study on TRUB2 function based on bacterial TruB/psi55, a homolog of TRUB2 [144].

4.6 Other PP1c Interaction Partners

Asporin (PIGR)

Asporin inhibits chondrogenesis and blocks TGF- β 1-induced expression of matrix genes and the resulting chondrocyte phenotypes [145].

Polymeric immunoglobulin receptor (PIGR)

This polymeric immunoglobulin receptor is normally transferred from the Golgi to the basolateral surface in epithelial cells and shows function in immune system by transporting polymeric IgA and IgM to the apical surface.

LIM and cysteine-rich domains protein 1 (LMCD1)

LMCD1 is regulated by insulin in human skeletal muscle [89]. It interacts with TGF β 1 [146]. In cardiomyocytes, LMCD1 combines with Dyx1c1 to form a complex, which will activate PP3 [147] or GATA6 [148] to regulate transcription factors. Since it interacts with PP3, another member of PPP family, it is not surprise to see it interacts with PP1. LMCD1 contains LIM domain, indicating that multiple functions may associate with LMCD1: actin regulation, Integrin regulation, and Cell-fate decision [149].

Lactotransferrin (LTF)

LTF is involved in muscle hypertrophy and myogenesis [150]. Originally, it was consider as a secretory iron-binding protein that can inhibit bacteria growth [151].

4.7 Influence of Muscle Fiber-type Composition

It is reported that type I, type IIa, and type IIx muscle fibers exist in human skeletal muscle [152], and significant variability has been shown between different ages [153], sexes [154], and individuals [153] [154]. Type IIx and IIa fibers are fast twitch fibers and type I fibers are slow twitch fibers. Proportion of fast and slow twitch fibers correlate with glucose metabolism and muscle insulin sensitivity [155], and it is possible that differences in muscle fiber type may lead

to unexplained variability in the protein interaction partners in the muscle biopsy samples.

Therefore, we plan to measure muscle fiber composition as described in the manuscript [156] for future studies. By assessing skeletal muscle fiber-type composition, we will be able to determine whether any observed differences in our major outcomes (e.g., differences in PP1c interaction partners) are related to differences in muscle fiber composition.

4.8 Summary

In this study, 46 proteins have been identified as PP1c interaction partners in human skeletal muscle in lean, healthy participants. And 8 of them express significant change after insulin stimulation (including ASPN, PIGR, POLR2A, PPP1R3A, PPP1R3B, PPP1R8, PPP1R12B, and RPS9). This study provides a list of PP1c interaction partners as reference for future studies in phosphatase biology, in interactome analysis, and in diabetes research. The successive step of this study will be validating of these interaction partners, and studying those partners showed significant changes upon insulin stimulation to determine whether insulin responsiveness of these PP1c partners would be diminished in type 2 diabetic patients.

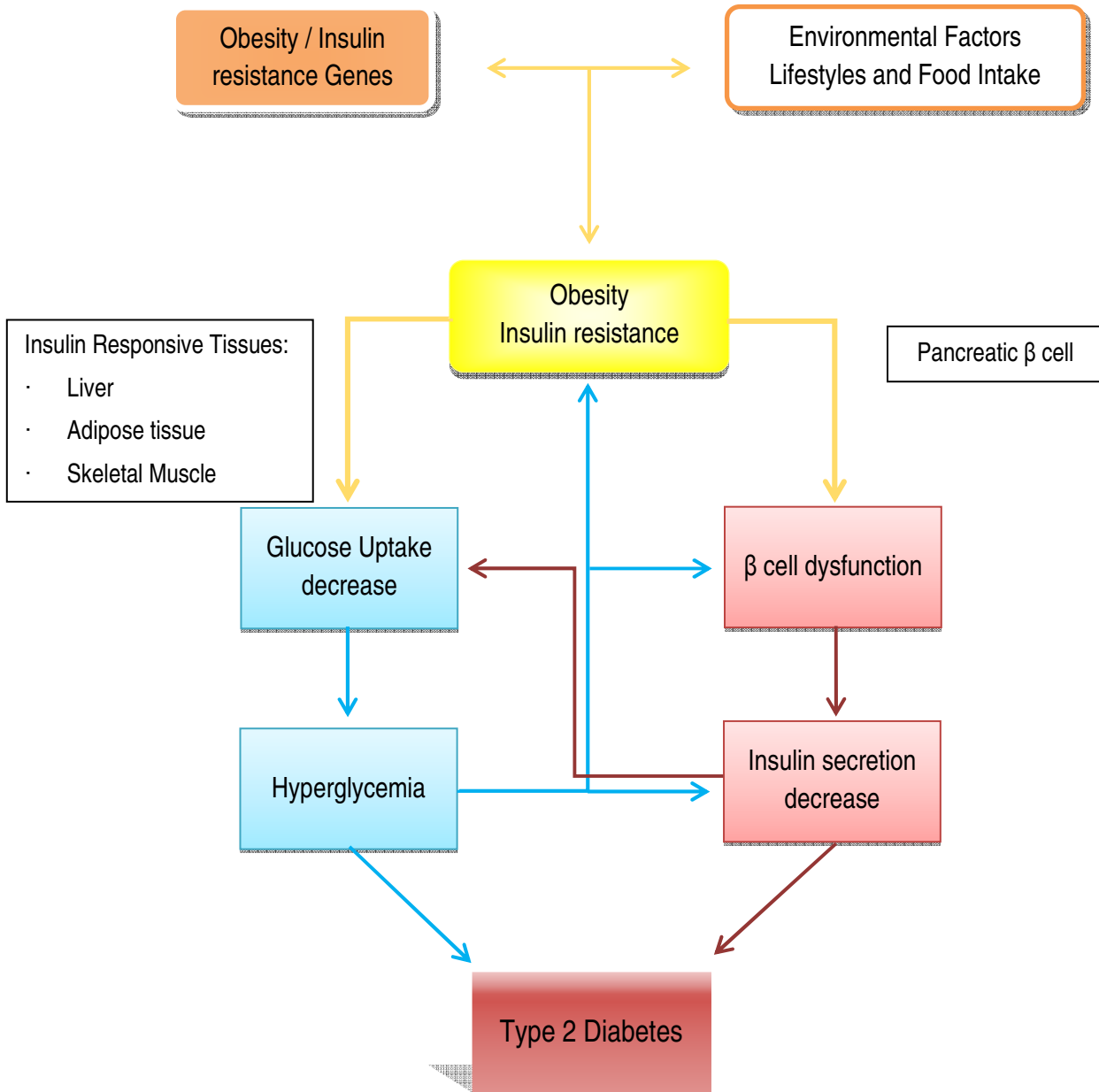


Figure 1. The causal relationship and tissue involvement of Type 2 Diabetes development.

Mutual interaction between genes and environmental factors (food intake and exercise habits) is a decisive factor of body shape and insulin sensitivity. Insulin resistance in insulin responsive tissues affects glucose uptake rate, resulting in the blood glucose increase. Hyperglycemia, as well as insulin resistance will progressively damage β cells, eventually leading to Type 2 Diabetes.

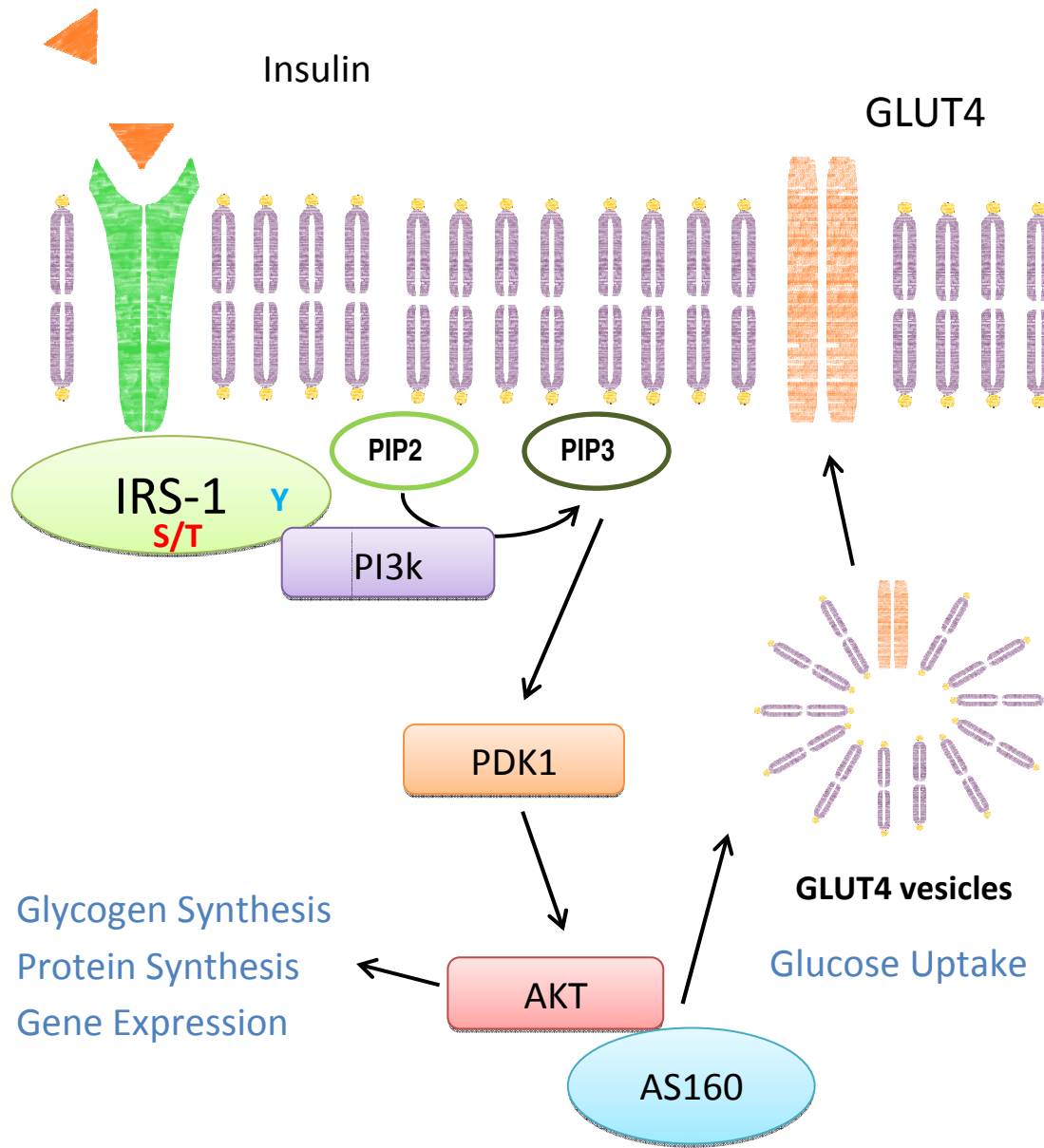


Figure 2. Overview of signal molecules involved in insulin signaling pathway.

Binding of insulin with insulin receptor initiates PI3k pathway, mediated by IRS-1 in skeletal muscle. PI3k pathway activates AKT (PKB), a molecule participates in multiple functions, including glucose uptake.

A

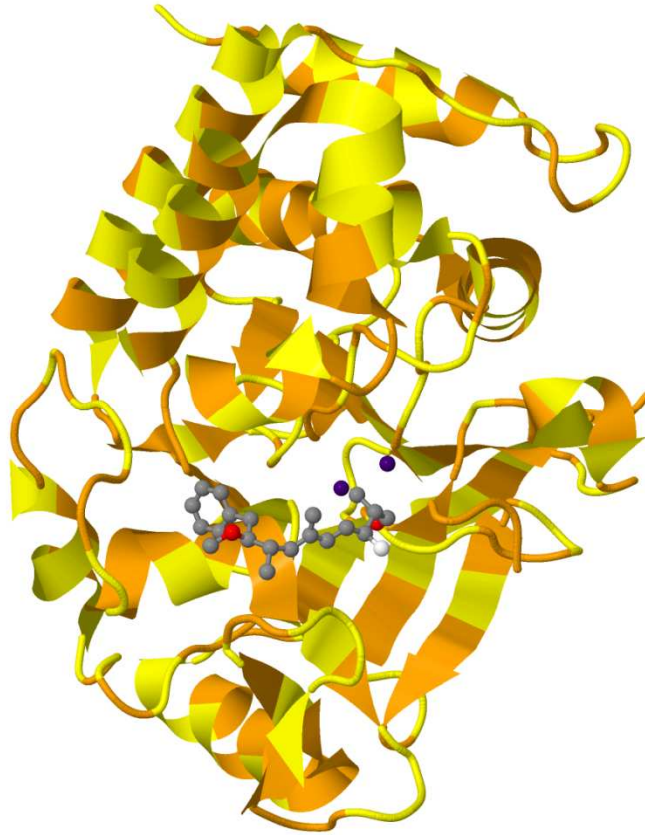


Figure 4. Structure information of PP1c

A.3D structure of human Protein Phosphatase 1 catalytic subunit, isoform α . Structure showed in ribbons indicates hydrophobicity - hydrophilic amino acids (yellow) and hydrophobic amino acids (orange).Catalytic core has been shown in atoms: Carbon (grey), Oxigen (red), Nitrogen (white), and Manganese (purple). Note that two Mn ions are critical for catalytic activity of PP1c. Structure information came from [157]. The figure creates by Jmol: an open-source Java viewer for chemical structures in 3D (<http://www.jmol.org/>);

B

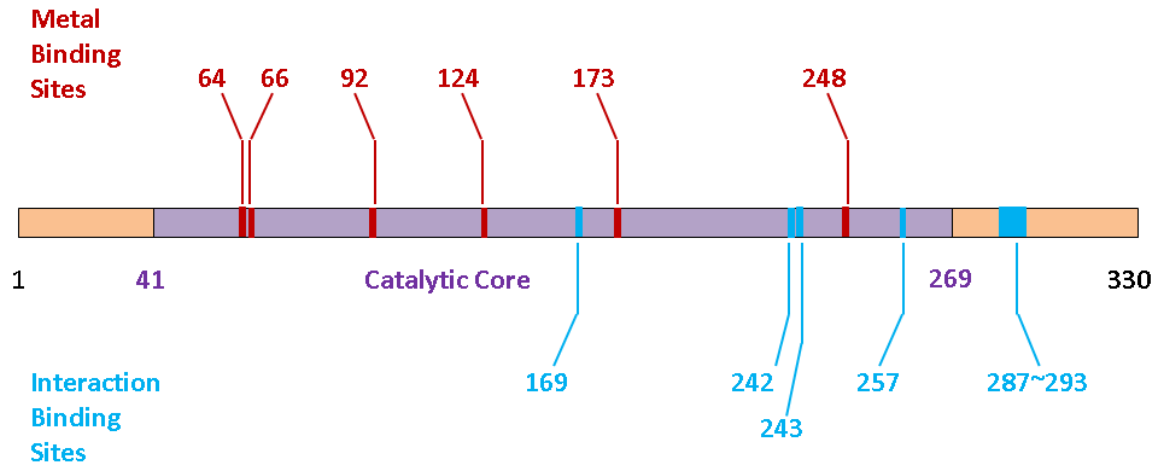


Figure 4. Structures of PP1c

B. Motif analysis of PP1c. Catalytic core is illustrated in purple; Metal binding sites are illustrated in red; and Interaction binding sites are illustrated in blue. Number of amino acid in sequence marked in the figure [30, 32, 33].

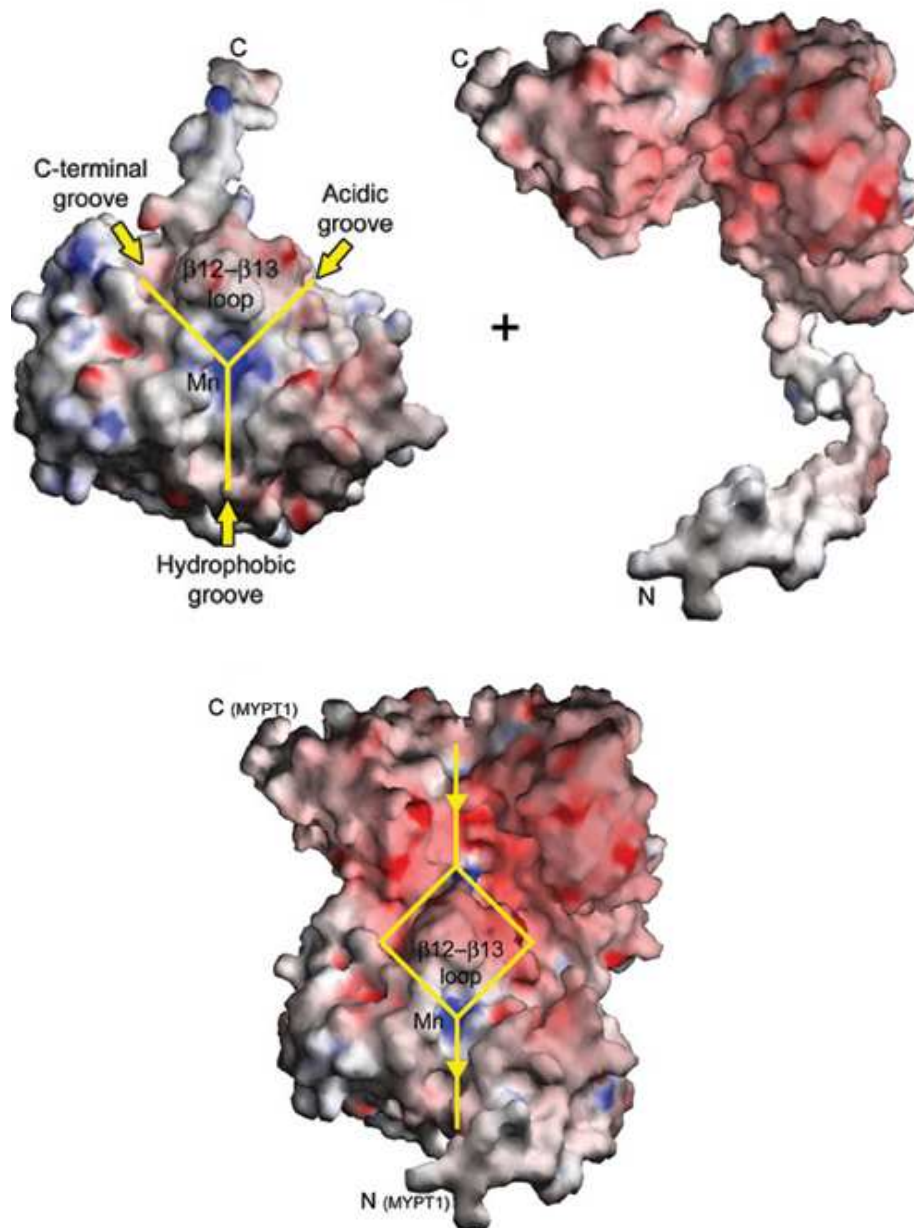


Figure 5. Interactions between PP1c and its interaction partner, MYPT1 to form a PP1 holoenzyme.

PP1c δ (up left) and PP1 regulatory subunit (up right) forms a complex (down) to achieve substrate specificity. The confluence of three binding groove (yellow lines) is catalytic core of PP1c. Reproduced with permission from [158].

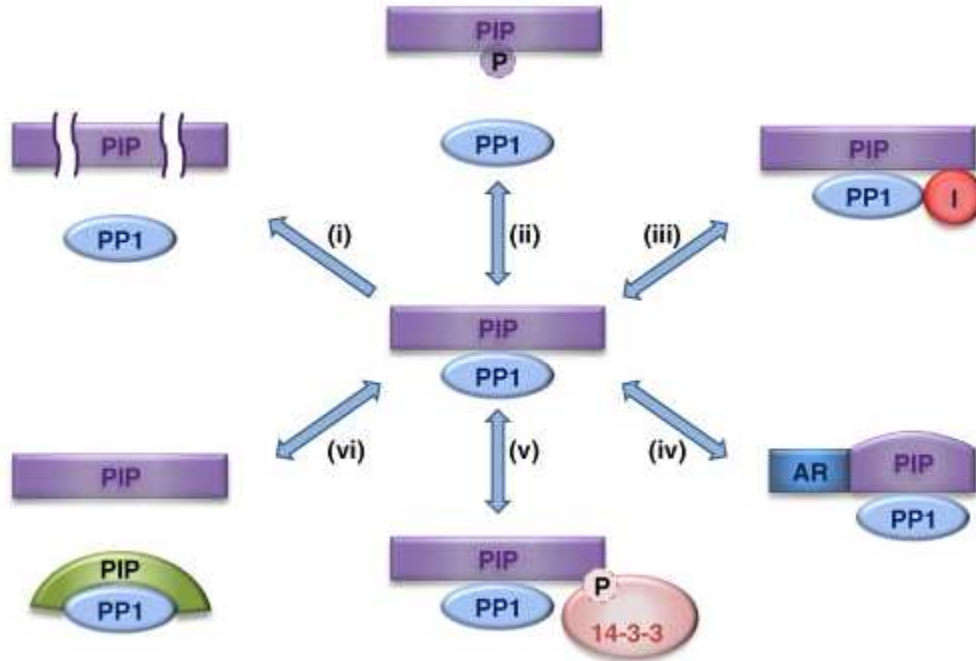


Figure 6. Regulation patterns of interaction partners on PP1c

Mechanisms by which interaction partners interact with PP1c include: (i) control PIP proteolysis; (ii) phosphorylation state affects association; (iii) Recruitment of inhibitors; (iv) Allosteric regulation; (v) Binding with 14-3-3 protein masked substrates; (vi) Competition for the same binding sites. Reproduced with permission from [31].

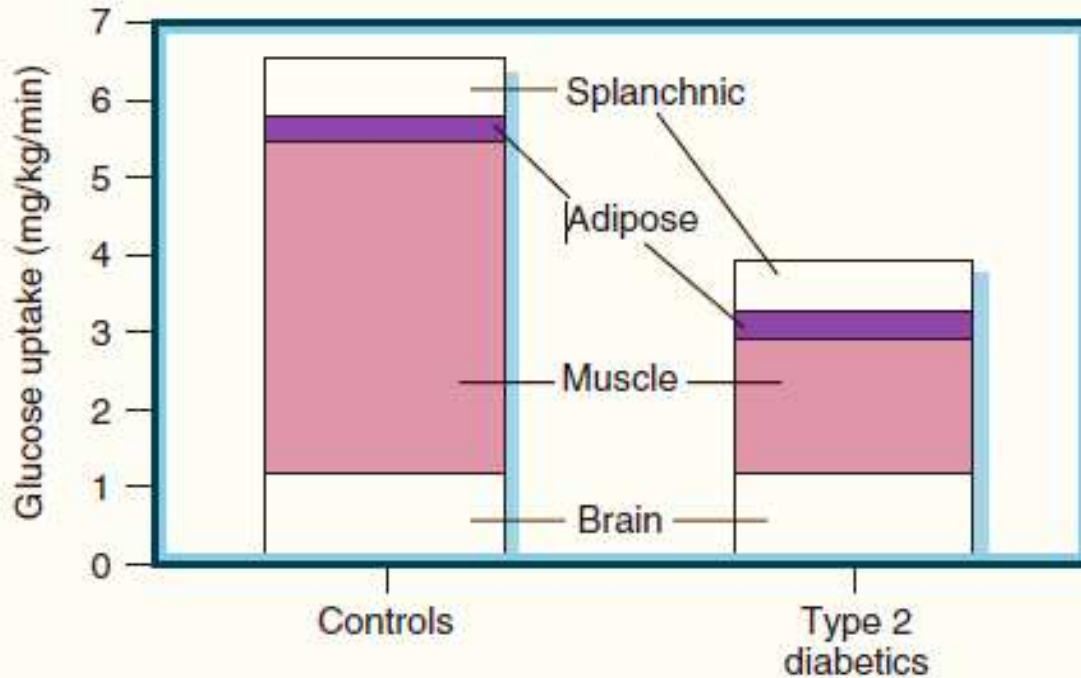


Figure 7. Summary of glucose metabolism change in Type 2 diabetes among different tissues during euglycemic insulin clamp studies.

Net glucose uptake is similar (Liver and Adipose tissue) or unaffected by hyperinsulinemia between healthy and T2D individuals. Muscle glucose uptake in healthy individuals accounts for approximately 75%–80% of total glucose uptake. In T2D patients, the most remarkable reduction in insulin-mediated glucose uptake is observed in muscle glucose disposal. Reproduced with permission from [159].

Figure 8A

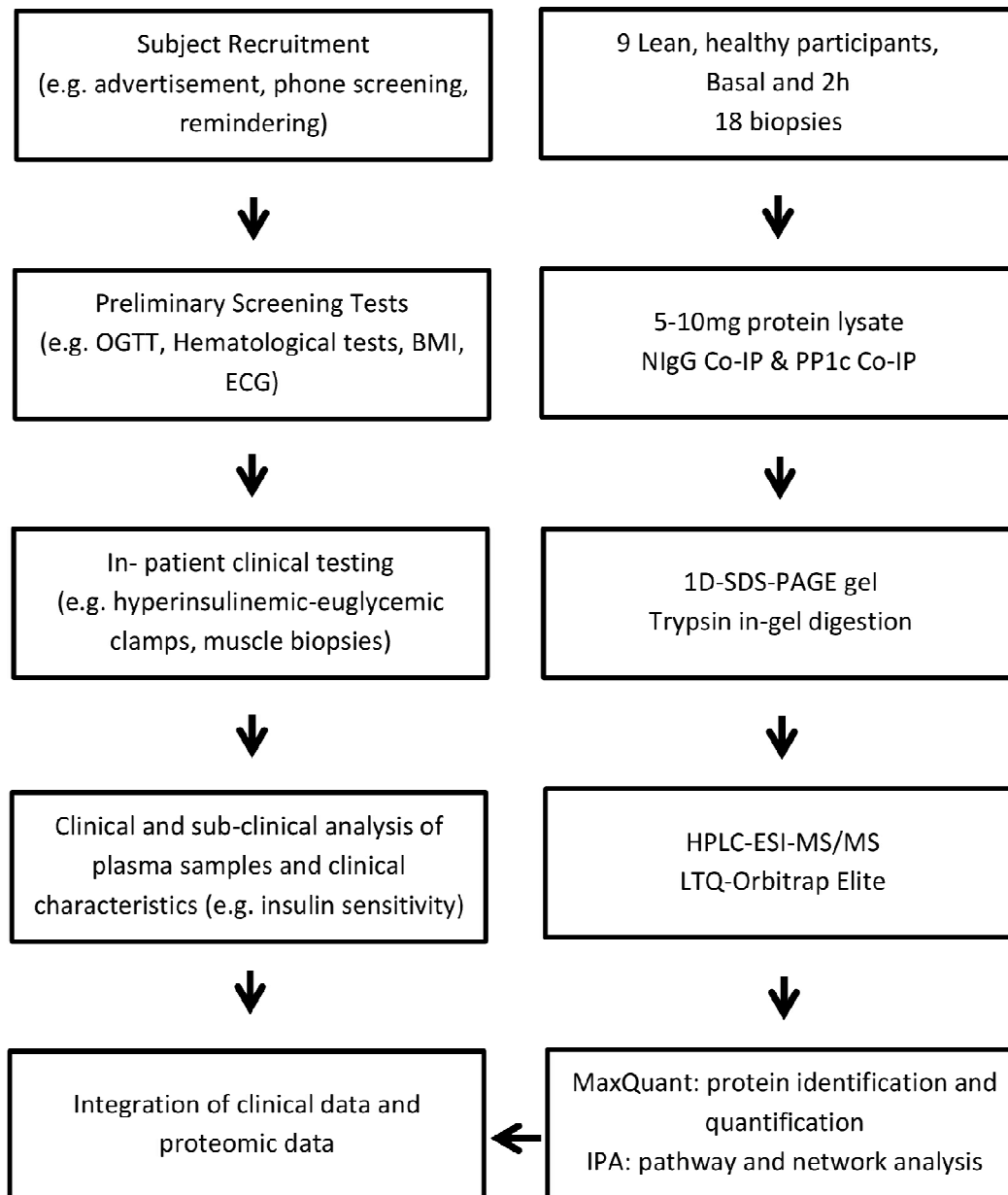


Figure 8A. General flow chart of clinical and proteomics data acquisition and analysis;

Figure 8B

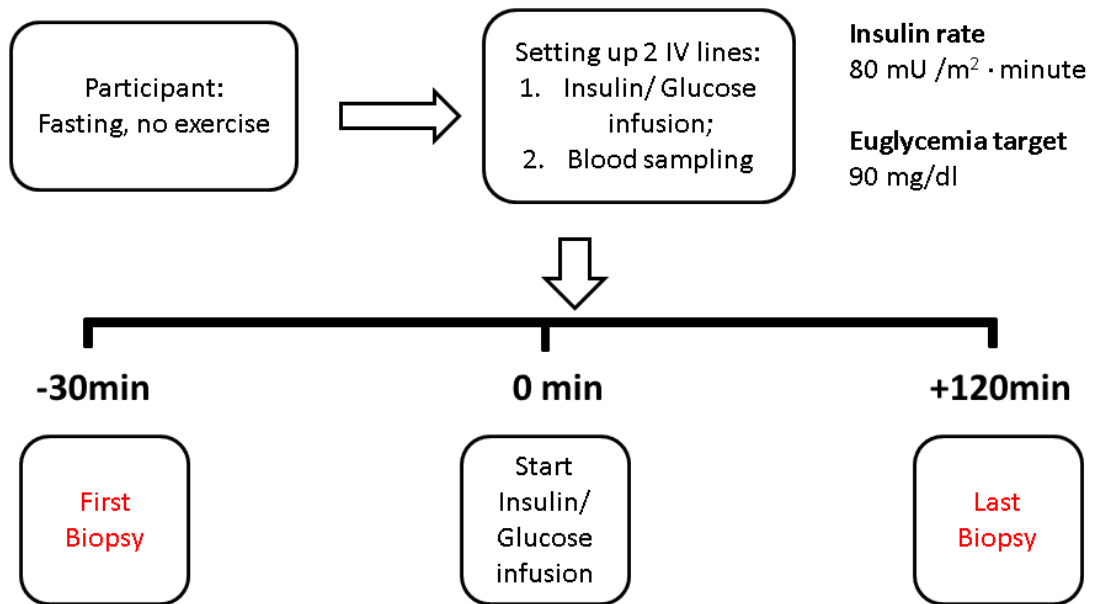


Figure 8B. Detailed illustration of clinical visit2

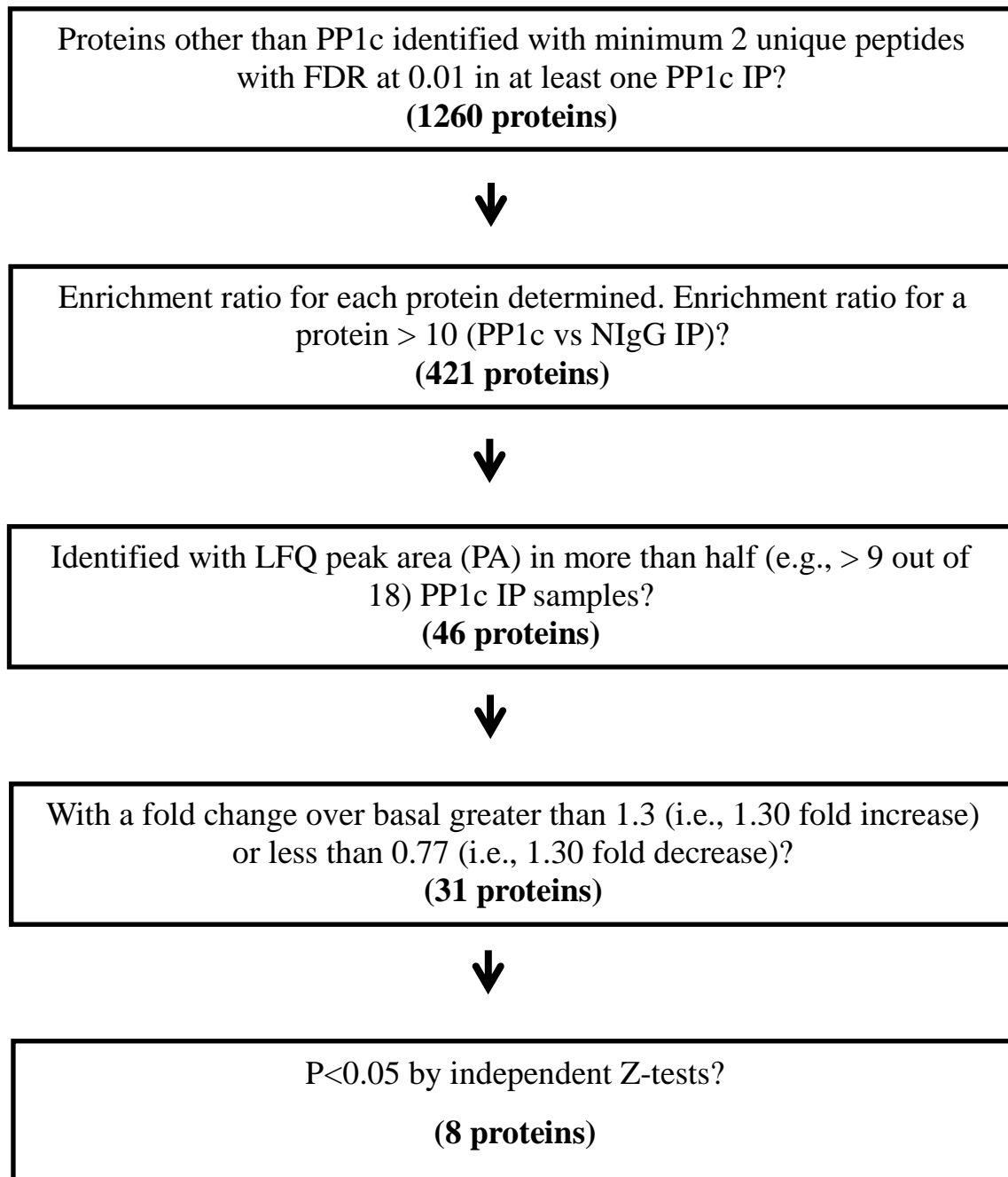


Figure 9. Detailed Proteomic Analysis workflow

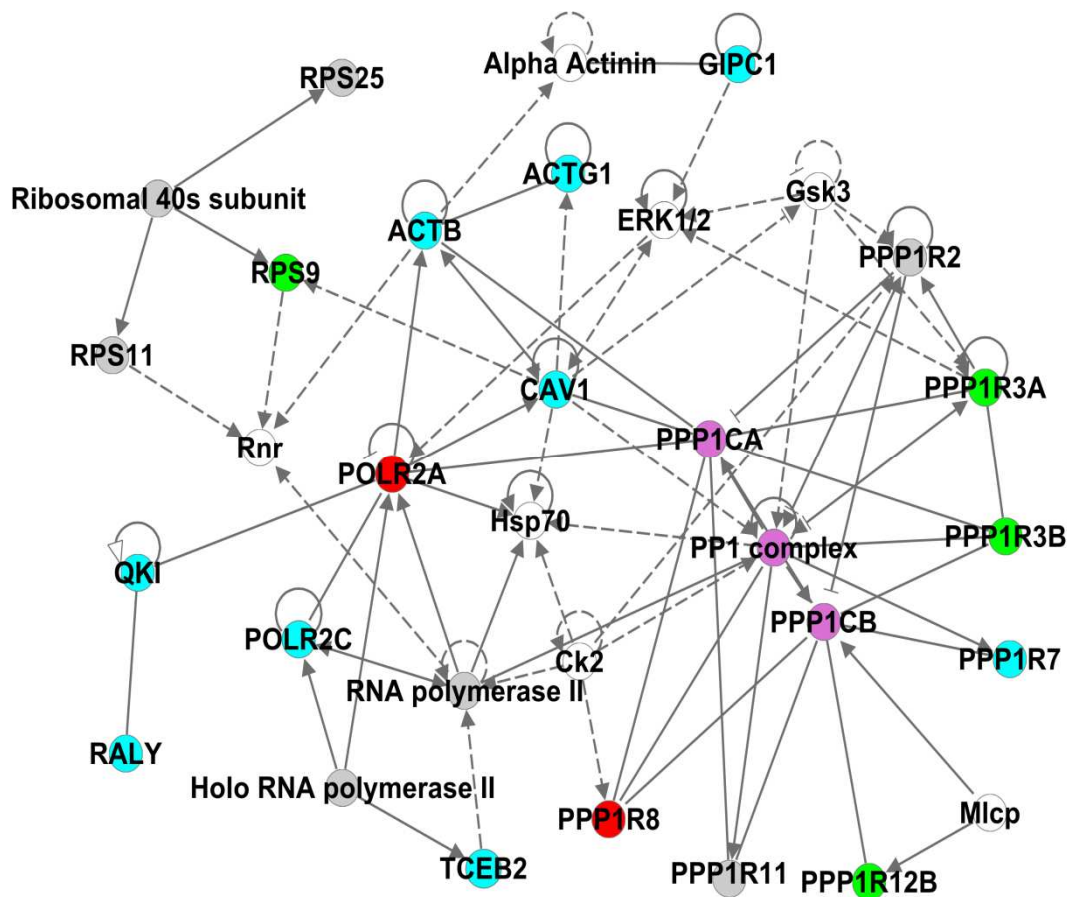


Figure 10. Network analysis of enriched PP1c interaction partners in human skeletal muscle revealed by proteomics and Ingenuity Pathway Analysis.

All parameters were set as default, with the exception of the number of molecules per network was maximized to 35. The top scored network (score at 51), which is related to Carbohydrate Metabolism, was shown, which contained 21 molecules derived from the list of 46 interaction partners identified in this study. PP1c is highlighted in purple, PP1c partners with 1.3 fold increased interaction with PP1c upon insulin-infusion are highlighted in green (significant increase, $P < 0.05$) or blue (insignificant increase, $P \geq 0.05$), partners significantly decreased are highlighted in red, and partners shows no change in this study are highlighted in grey, respectively. Solid and dashed connecting lines indicate the presence of direct and indirect interactions in the Ingenuity database, respectively.

Figure 11A

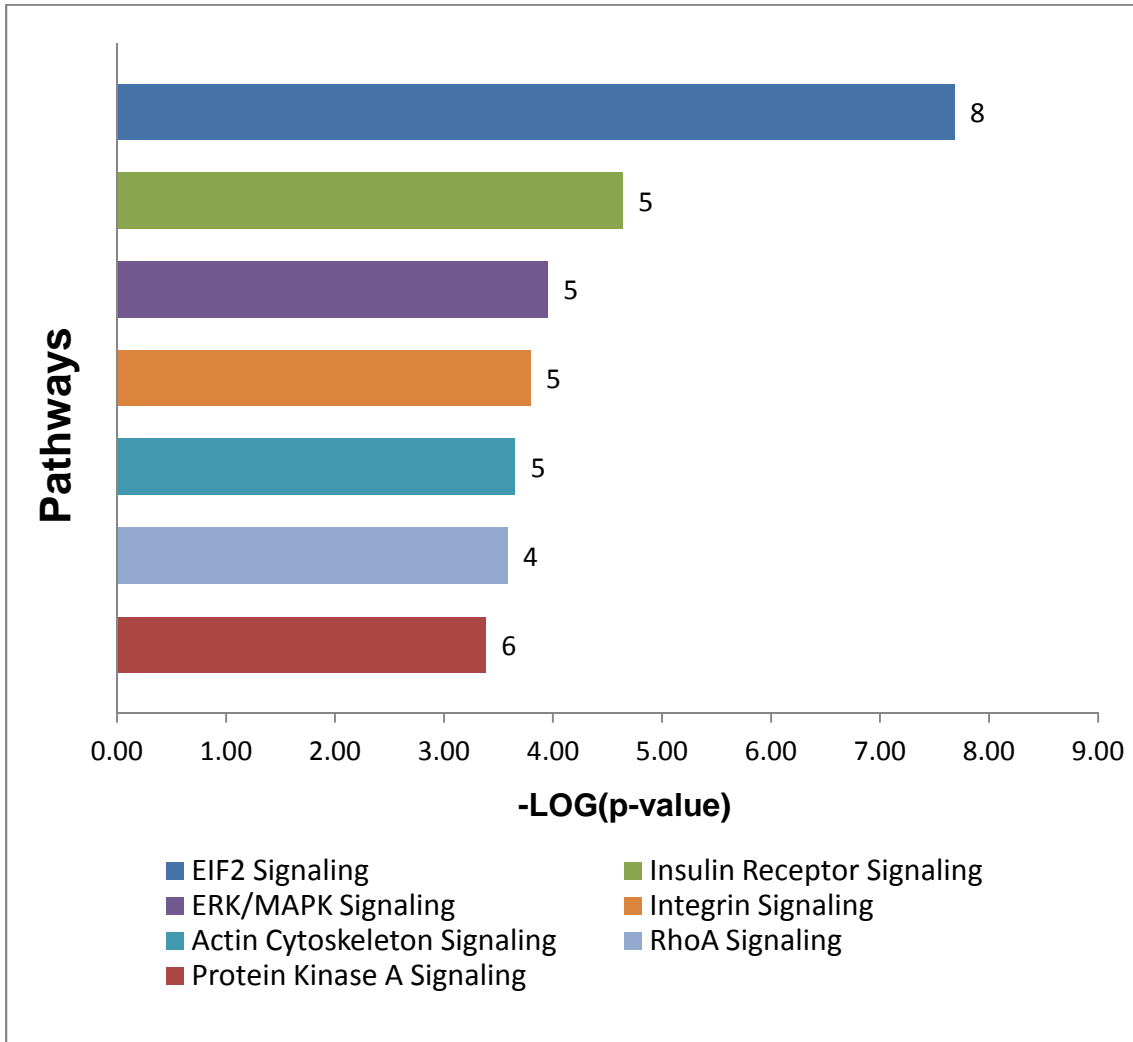


Figure 11. Significantly enriched pathways for the PP1c interaction partners identified in this study revealed by Ingenuity Pathways Analysis. A). Significantly enriched pathways. The total number of identified PP1c interaction partners for a given pathway in this study is denoted beside each bar. B) PP1c interaction partners in EIF2 Signaling.

Figure 11B

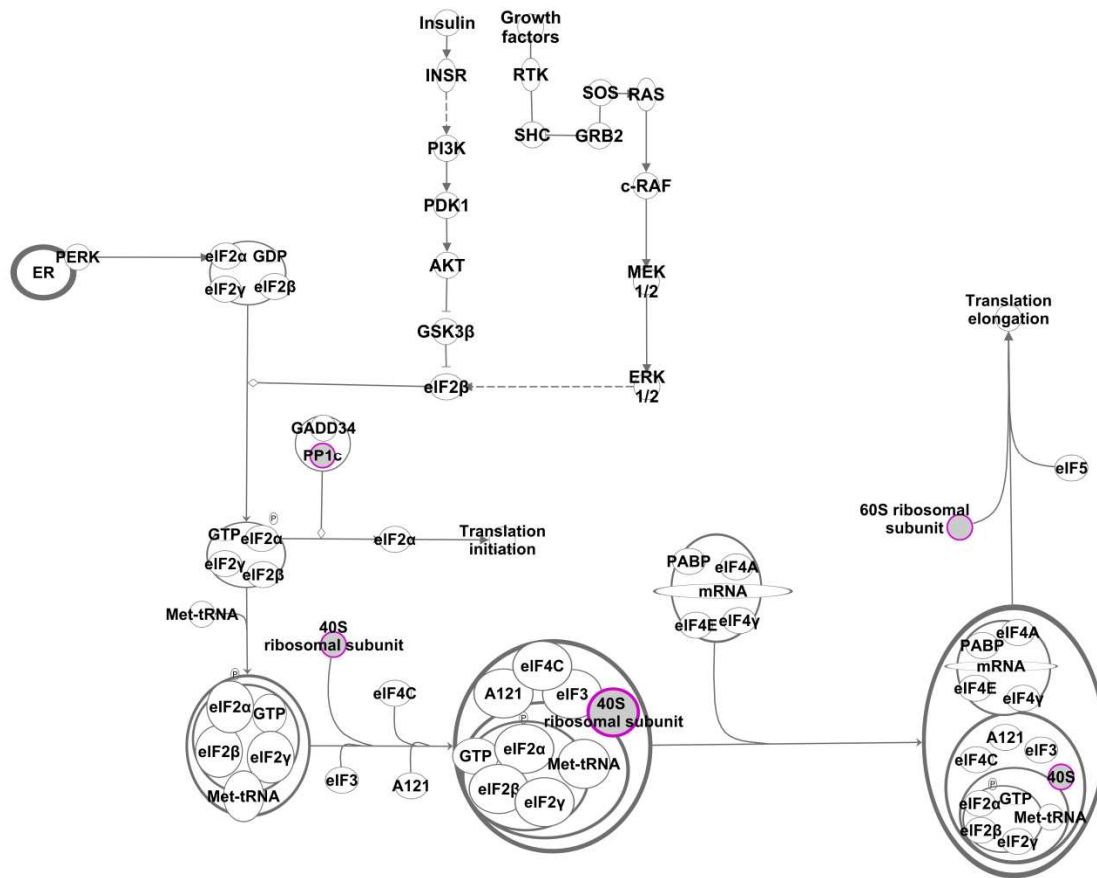


Figure 11B. PP1c interaction partners in EIF2 Signaling

Table 1. Clinical characteristics participants in the PP1c interaction partner study.

Results were shown as mean \pm SEM. Normal values are in **Bold** after BMI, 2h OGTT glucose, HBA1c, and Fasting plasma glucose values.

	9 participants in lean group
Gender (M/F)	(5/4)
Age (years)	35.9 \pm 3.3
BMI (kg/m^2)	23.8 \pm 0.7 (< 25)
2h OGTT Glucose (mmol/l)	5.5 \pm 0.3
2h OGTT Glucose (mg/dl)	98.7 \pm 6.1 (<140)
HBA1c (%)	5.2 \pm 0.1 (<5.7)
Fasting plasma glucose (mmol/l)	4.5 \pm 0.1
Fasting plasma glucose (mg/dl)	81.2 \pm 1.7 (<100)
Fasting plasma insulin (pmol/l)	29.8 \pm 2.9
M-value (mg/kg/min)	8.8 \pm 1.0

Table 2 The 46 proteins/ protein groups met the 2 rigorous criteria (See Methods for details) for classification as PP1c interaction partners in human skeletal muscle.

Protein	Gene names	mean fold change over basal
Asporin	ASPN	3.27±1.10*
Polymeric immunoglobulin receptor	PIGR	5.59±1.79*
Protein phosphatase 1 regulatory subunit 3A	PPP1R3A	1.36±0.15*
Protein phosphatase 1 regulatory subunit 3B	PPP1R3B	1.43±0.20*
Protein phosphatase 1 regulatory subunit 12B	PPP1R12B	1.52±0.26*
40S ribosomal protein S9	RPS9	1.89±0.41*
Isobutyryl-CoA dehydrogenase, mitochondrial	ACAD8	3.89±1.78
Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADVL	2.35±1.32
Actin, cytoplasmic	ACTB;ACTG1	6.03±4.54
3-methyl-2-oxobutanoate dehydrogenase kinase, mitochondrial	BCKDK	1.88±0.69
Caveolin-1	CAV1	2.66±1.40
Cystatin-B	CSTB	1.32±0.57
Prothrombin	F2	1.67±0.40
PDZ domain-containing protein GIPC1	GIPC1	1.96±0.77

Protein	Gene names	mean fold change over basal
LIM and calponin homology domains-containing protein 1	LIMCH1	1.96±0.92
Lactotransferrin;Kaliocin-1	LTF	1.70±0.96
39S ribosomal protein L49	MRPL49	1.39±0.49
DNA-directed RNA polymerase II subunit RPB3	POLR2C	2.95±1.56
Protein phosphatase 1 regulatory subunit 7	PPP1R7	1.50±0.53
Pentatricopeptide repeat-containing protein 1	PTCD1	1.39±0.51
Protein quaking	QKI	1.34±0.32
RNA-binding protein Raly	RALY	1.30±0.51
60S ribosomal protein L18a	RPL18A	8.05±6.87
60S ribosomal protein L3-like	RPL3L	3.21±1.68
Semenogelin-1;Semenogelin-2	SEMG1;SEMG2	1.37±0.59
Transcription elongation factor B polypeptide 2	TCEB2	1.43±0.39
Mitochondrial import inner membrane translocase subunit TIM50	TIMM50	1.68±1.12
TRIO and F-actin-binding protein	TRIOBP	1.64±0.34
Uveal autoantigen with coiled-coil domains and ankyrin repeats	UACA	1.52±0.41

Protein	Gene names	mean fold change over basal
DNA-directed RNA polymerase II subunit RPB1	POLR2A	0.72±0.13*
Nuclear inhibitor of protein phosphatase 1	PPP1R8	0.54±0.13*
LIM and cysteine-rich domains protein 1	LMCD1	0.59±0.26
Estradiol 17-beta-dehydrogenase 8	HSD17B8	1.01±0.38
Metaxin-2	MTX2	0.95±0.24
Phosphatase and actin regulator 4	PHACTR4	1.04±0.27
Prolactin-inducible protein	PIP	0.99±0.39
Protein phosphatase 1 regulatory subunit 11	PPP1R11	1.22±0.17
Protein phosphatase inhibitor 2	PPP1R2;PPP1R2P3	1.04±0.21
60S ribosomal protein L14	RPL14	1.00±0.48
40S ribosomal protein S11	RPS11	1.01±0.25
40S ribosomal protein S25	RPS25	0.79±0.20
Splicing factor 3B subunit 1	SF3B1	1.25±0.54
Double-stranded RNA-binding protein Staufien homolog 2	STAU2	0.91±0.26
Erythrocyte band 7 integral membrane protein	STOM	1.28±0.45
Heterogeneous nuclear ribonucleoprotein Q	SYNCRIP	1.18±0.44

Protein	Gene names	mean fold change over basal
Transformer-2 protein homolog beta	TRA2B	1.29±0.56
Probable tRNA pseudouridine synthase 2	TRUB2	1.08±0.24

Results were shown as mean fold change over basal \pm SEM. A 2-fold change indicates a 2-fold increase, while a 0.5 fold-change indicates a 2-fold decrease. *, $P < 0.05$ vs. basal. Proteins highlighted in green are PP1c interaction partners showed a significant increase upon insulin-infusion in humans (>1.3 fold vs. basal, $P < 0.05$). Proteins highlighted in green are PP1c interaction partners showed an insignificant increase upon insulin-infusion (>1.3 fold vs. basal, $P \geq 0.05$). Proteins highlighted in red are PP1c interaction partners showed a significant decrease upon insulin-infusion (>1.3 fold decrease vs. basal or <0.77 fold change over basal, $P < 0.05$). Proteins highlighted in orange are PP1c interaction partners showed an insignificant decrease upon insulin-infusion (>1.3 fold decrease vs. basal or <0.77 fold change over basal, $P \geq 0.05$). Proteins highlighted in yellow are PP1c interaction partners showed less than 1.3 fold change upon insulin-infusion.

Table 3 Significantly enriched pathways for the PP1c interaction partners identified in the study revealed by Ingenuity Pathways Analysis.

Ingenuity Canonical Pathways	$-\log(p\text{-value})$	Gene Names	Number of identified PP1c interaction partners in the study
EIF2 Signaling	7.68	RPL14,RPL18A,RPS9,PPP1CB,RPL3L,RPS25,PPP1CA,RPS11	8
Protein Kinase A Signaling	3.38	PPP1R7,TIMM50,PPP1CB,PPP1R11,PPP1R3A,PPP1CA	6
Insulin Receptor Signaling	4.64	PPP1R7,PPP1CB,PPP1R11,PPP1R3A,PPP1CA	5
ERK/MAPK Signaling	3.95	PPP1R7,PPP1CB,PPP1R11,PPP1R3A,PPP1CA	5
Integrin Signaling	3.79	PPP1R12B,ACTB,CAV1,PPP1CB,ACTG1	5
Actin Cytoskeleton Signaling	3.65	PPP1R12B,ACTB,PPP1CB,ACTG1,F2	5
RhoA Signaling	3.58	PPP1R12B,ACTB,PPP1CB,ACTG1	4

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ABSTRACT**PROTEOMIC DISCOVERY OF PROTEIN PHOSPHATASE 1 CATALYTIC
SUBUNIT PROTEIN INTERACTION PARTNERS IN HUMAN SKELETAL
MUSCLE**

by

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Protein Phosphatase 1 (PP1), a member of Serine/ Threonine Phosphatase family, targets its dephosphorylation activity on serine and threonine residues. As the catalytic subunit of PP1, PP1c can achieve its substrate specificity only by binding with PP1 regulatory subunits. Previous researches have shown that PP1c can involve in multiple functional regulation by associating with various interaction partners. Since serine/ threonine phosphorylation on the Insulin receptor substrate-1 (IRS1) may direct inactivation and degradation of IRS1, this phosphorylation activity is believed to be a source of Insulin Resistance. PP1 is hypothesized to dephosphorylate serine/ threonine site on IRS1, which may rescue the Insulin resistance.

However, the PP1c interaction partners involve in this process is unclear, especially in vivo in humans. In the current work, we explored PP1c complexes in skeletal muscle biopsies from lean healthy participants obtained before insulin

infusion and after 2 hour insulin infusion, using the proteomic approach developed in our laboratory. We identified 46 previously unreported endogenous PP1c interaction partners, which is the largest PP1c interactome in human skeletal muscle. These novel PP1c interaction partners may serve as new targets to investigate PP1c complexes in different diseases. Furthermore, we identified 8 proteins show significantly changes after insulin treatment. These novel PP1c interactions provide new insights into the molecular mechanism of insulin action and identify new targets for further PP1c researches in Type 2 Diabetes.

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