Effect of Insulin and Pioglitazone on Protein Phosphatase 2A Interaction Partners in Primary Human Skeletal Muscle Cells Derived from Obese Insulin-Resistant Participants

Lana Alghanem,$§ Xiangmin Zhang,$§ Ruchi Jaiswal, Berhane Seyoum, Abdullah Mallisho, Zaher Msallaty, and Zhengping Yi*

Cite This: ACS Omega 2022, 7, 42763−42773

ABSTRACT: Skeletal muscle insulin resistance is a major contributor to type-2 diabetes (T2D). Pioglitazone is a potent insulin sensitizer of peripheral tissues by targeting peroxisome proliferator-activated receptor gamma. Pioglitazone has been reported to protect skeletal muscle cells from lipotoxicity by promoting fatty acid mobilization and insulin signaling. However, it is unclear whether pioglitazone increases insulin sensitivity through changes in protein−protein interactions involving protein phosphatase 2A (PP2A). PP2A regulates various cell signaling pathways such as insulin signaling. Interaction of the catalytic subunit of PP2A (PP2Ac) with protein partners is required for PP2A specificity and activity. Little is known about PP2Ac partners in primary human skeletal muscle cells derived from lean insulin-sensitive (Lean) and obese insulin-resistant (OIR) participants. We utilized a proteomics method to identify PP2Ac interaction partners in skeletal muscle cells derived from Lean and OIR participants, with or without insulin and pioglitazone treatments. In this study, 216 PP2Ac interaction partners were identified. Furthermore, 26 PP2Ac partners exhibited significant differences in their interaction with PP2Ac upon insulin treatments between the two groups. Multiple pathways and molecular functions are significantly enriched for these 26 interaction partners, such as nonsense-mediated decay, metabolism of RNA, RNA binding, and protein binding. Interestingly, pioglitazone restored some of these abnormalities. These results provide differential PP2Ac complexes in Lean and OIR in response to insulin/pioglitazone, which may help understand molecular mechanisms underpinning insulin resistance and the insulin-sensitizing effects of pioglitazone treatments, providing multiple targets in various pathways to reverse insulin resistance and prevent and/or manage T2D with less drug side effects.

INTRODUCTION

Type-2 diabetes (T2D) accounts for >90% of all diabetic cases and is a consequence of the combination of insulin resistance and relative insulin deficiency. Majority (>70%) of the insulin-stimulated glucose uptake occurs in skeletal muscle, and skeletal muscle insulin resistance is among primary contributors to the development of T2D. Multiple defects have been reported to contribute to the etiology of skeletal muscle insulin resistance, such as impairments in insulin receptor substrate 1 (IRS-1) protein tyrosine phosphorylation, PI-3 kinase and AKT activation as well as glucose uptake, accumulation of free fatty acids (FFA) in plasma and high intramyocellular fat content, defects in oxidative phosphorylation processes in mitochondria and generation of reactive oxygen species. On the other hand, hyperglycemia in T2D patients may worsen insulin resistance.

T2D is being treated with a variety of medications, including pioglitazone (Pio), a potent insulin sensitizer for skeletal muscle, belonging to the thiazolidinedione family. However,
the disclosure of adverse effects limits the clinical prescription of Pio.21 Pio is a synthetic ligand for the peroxisome proliferator-activated receptor gamma (PPAR-γ), a nuclear receptor regulating lipid and glucose metabolism gene expression and insulin sensitivity of peripheral tissues.21−26

One of the main actions of Pio was to increase lipid storage, preventing the accumulation of FFAs in circulation, and preventing FFA-induced insulin resistance.27,28 Pio has been shown to improve tyrosine phosphorylation of IR and IRS and enhance P3 kinase activity in C2C12 skeletal muscle cells.29

In addition, Pio increased adiponectin levels and muscle AMPK signaling, which augmented muscle glucose uptake and fatty acid oxidation in human muscle from T2D patients.30 Moreover, Bajpeyi et al. have demonstrated that 12 week Pio treatment in diabetic patients increased insulin sensitivity possibly through the redistribution of lipid content in muscles.30 More recently, Pio treatment was shown to increase abundance of multiple mitochondrial proteins in oxidative phosphorylation while decreasing abundance of certain mitochondrial proteins in fatty acid catabolism in skeletal muscle of T2D patients.31 Nonetheless, additional studies are needed to better understand the molecular actions of Pio that sensitize muscle to insulin.

In skeletal muscle, protein phosphorylation events are important in regulating various signaling pathways, including insulin signaling. Abnormalities in these processes are the main cause in the development of skeletal muscle insulin resistance.32−34 Most research on the regulation of these events has been focused on kinases, and more research on phosphatases, such as protein phosphatase 2A (PP2A), is needed.

PP2A is one of the major serine/threonine phosphatases, regulating many fundamental cellular events, including proliferation, survival, and insulin signaling.35−37 PP2A can inactivate multiple kinases (e.g., AKT, PKC, and p70S6 kinase) and activate other kinases (e.g., GSK-3 and Casein kinase I).35,36 Besides, the catalytic subunit of PP2A (PP2Ac) was reported to interact with IRS1 in cardiomyocytes,38 and its interaction with IRS1 is higher in the skeletal muscle of obese controls and T2D participants compared to lean controls.39 It is unclear whether PP2A plays a role in Pio’s action in human skeletal muscle (HSKM) cells.

PP2A is found in all major subcellular compartments and is widely expressed in various types of cells and tissues (e.g., islet, liver, skeletal muscle, and so on.).35,40 PP2A is a protein complex, existing in either heterodimeric [A/C] or trimeric [A/B/C] conformation comprising the scaffolding A subunit (PP2Aa), the regulatory B (PP2Ab), and PP2Ac, the catalytic subunit. PP2Ac has two isoforms, PP2Acα and PP2Acβ, which share a 97% homology.41 PP2Acα-deficient mice are embryonic lethal,42 and PP2Acα knocked out in primordial germ cells caused mouse infertility.43 PP2Acβ liver-specific knockout mice have improved glucose metabolism and insulin sensitivity.44

PP2Ac is fairly non-specific and may dephosphorylate a large number of substrates.45 The interaction of PP2Ac with PP2Aa and PP2Ab is required to regulate PP2A function, specificity, and subcellular localization.35,40 Surprisingly, little is known about the PP2Ab as well as PP2A substrates and other regulators in primary HSKM cells derived from obese insulin-resistant participants.

Co-immunoprecipitation (Co-IP) followed by mass spectrometry-based proteomics is an effective approach to characterize protein−protein interactions.39,46−49 However, protein overexpression and/or epitope-tagged bait proteins are widely used. We have developed a straightforward, label-free approach to quantify differences in the abundance of endogenous proteins interacting with a bait protein.48 Using this approach, we identified new IRS1 interaction partners in the skeletal muscle from lean controls, obese controls and T2D participants,49 insulin-responsive Akt2 interaction partners in L6 cells,49 PP2Ac interaction partners in INS-1 832/13 rat pancreatic beta cells in response to high glucose,57 and Rac1 interaction partners upon high-glucose treatment in pancreatic beta-cells.56 In the present study, we applied this proteomic method to study endogenous PP2Ac interaction partners in primary HSKM cells derived from lean insulin-sensitive (Lean) and obese insulin-resistant (OIR) participants treated with insulin and/or Pio. The goals of the study were to (1) verify that proteins shown to interact with PP2Ac in other cell types indeed interact with PP2Ac in primary HSKM cells, (2)

Figure 1. Flowchart for clinical, biological, and proteomics workflows.
identify novel PP2Ac interaction partners in primary HSKM cells, (3) discover abnormalities in PP2Ac interactions in OIR compared to Lean, and (4) determine whether Pio treatment can reverse abnormalities in PP2Ac interactions in OIR and render them similar to Lean.

EXPERIMENTAL SECTION

The overall research design and methods are similar to the one described in our previous publication, which described the finding of new IRS1 complexes in HSKM in OIR/T2D, with the main exception that antibody specific to PP2Ac instead of IRS1 was used to pull-down PP2Ac and its interaction partners (Figure 1). A combination of clinical, biological, and proteomics data acquisition and data analysis was employed. The clinical studies included subject recruitment, comprehensive screening tests, and hyperinsulinemic-euglycemic clamp and muscle biopsies. The biological studies included primary cell culture, subculture, myotube differentiation, and treatment with Pio and insulin. The proteomics studies included primary cell culture, subculture, myotube differentiation, and treatment with Pio and insulin. The proteomics studies included cell homogenization; immunoprecipitation of the "bait" protein (endogenous PP2Ac); separation of interaction proteins by 1D-SDS-PAGE; generation of peptide fragments by in-gel trypsin digestion; and identification of co-immunoprecipitating proteins by UPLC-ESI-MS/MS analysis. Multiple biological comparisons and NIgG immunoprecipitation (as a non-specific control) were used to minimize false positives. Extensive bioinformatics and literature search were used to identify pathways/functions, which identified PP2Ac interaction partners were involved, that were impacted by insulin, insulin-resistance, and Pio.

Reagents. Protein A sepharose (Sigma, St Louis, MO), iodoacetamide (lot# SLB55004, SIGMA), C18 ZipTip, HPLC grade acetonitrile (ACN), trifluoroacetic acid and formic acid (FA), normal mouse IgG (NIgG), Pio hydrochloride, and PP2Ac mouse monoclonal antibody were from MilliporeSigma (Burlington, MA). Sequence-grade trypsin was from Promega and dithiothreitol (DTT) was from ThermoFisher (Waltham, MA).

Human Participants. This protocol was approved by the Institutional Review Board of Wayne State University. The clinical studies were carried out as described in our publications. Briefly, the clinical studies started with participant recruitment, followed by comprehensive screening tests (visit 1) and a hyperinsulinemic-euglycemic clamp to assess insulin sensitivity and expose skeletal muscle to insulin in vivo with muscle biopsies obtained (visit 2).

Primary HSKM Cell Culture and Treatments. HSKM cell cultures were carried out as previously described. In brief, the biopsy was washed and minced, followed by centrifugation and filtration. The resulting HSKM cells were cultured and maintained. Once the myoblasts reached 95% confluence in 10 cm Petri dishes, the growth medium was replaced with 10 ml of differentiation medium (DMEM containing 2% horse serum and 1% PSG) on the following day (designated as day 1). The cell cultures were incubated in a humidified atmosphere at 37 °C and 5% CO2. On day 3, the differentiation medium was discarded, and the cells were supplemented with 10 ml of fresh differentiation medium. On day 4, the cells were treated with or without 2 μM Pio for 24 h.

Myotubes were serum-starved for 4 h and immediately stimulated by 100 nM insulin for 15 min before harvesting. Therefore, for each participant, there were 4 cell plates: plate 1: no treatment, used as a control, BAS; plate 2: insulin 100 nM for 15 min, INS; plate 3: Pio 2 μM for 24 h, Pio_BAS; and plate 4: Pio 2 μM for 24 h plus insulin 100 nM for 15 min Pio_INS.

HPLC-ESI-MS/MS-Based Quantitative Proteomics Analysis. Proteomics analysis on cell lysates were performed as described in our articles and in Figure 1b,c. Briefly, four 10 cm cell plates from each of the 16 participants were analyzed (BAS, INS, Pio_BAS and Pio_INS). In total, 64 cell plates were lysed. For each sample, approximately 1 mg of total protein was separated by 1D-SDS-PAGE followed by TrypSAP digestion. The resulting peptide mixtures were then subjected to ESI-MS/MS analysis by LC-MS. Protein identification was performed using the Mascot search engine (http://www.matrixscience.com) with the human database. The following parameters were used: trypsin as the enzyme, allow one missed cleavage, fragment mass tolerance of 3.00 ppm, and peptide mass tolerance of 0.30 Da.
protein was first incubated with 4 μg of normal mouse IgG conjugated to protein A beads. Three hours later, beads were harvested and served as a nonspecific control. The precleared supernatant was further incubated with 4 μg of PP2Ac mouse monoclonal antibody conjugated to protein A beads. After overnight incubation, the beads were harvested.

Both NIgG and anti-PP2Ac beads were washed three times with lysis buffer. Subsequently, the beads were boiled in 50 μL of 2 × SDS buffer containing 5% mercaptoethanol at 95 °C for 5 min. Bead eluates were resolved by 10% 1D-SDS-PAGE. For each lane, three slices (250–75, 75–37, and 37–10 kDa) were excised, destained, and exposed to 50 mM DTT, followed by iodoacetamide (IAA) treatment, in-gel trypsin digestion, peptide purification by ZipTip Pipette tips, and UPLC-ESI-MS/MS analysis using an Orbitrap Fusion Lumos as described.15,52–54

The peptide mixture was separated with a gradient of 2–35% buffer B (100% ACN and 0.1% FA) in 70 min, starting with 2% B and increase to 5% in 1 min, to 25% B in 60 min, to 35% B in 10 min, to 90% B in 1 min, hold at 90% B for 5 min, and then 2% buffer B at a flow rate of 400 nL/min on a C18−reversed phase column (75 μm ID, 45 cm length) packed in-house with ReproSil-Pur C18-AQ μm resin (Dr. Maisch GmbH) in buffer A (0.1% FA). MS spectra were generated from Orbitrap Fusion Lumos (ThermoFisher) equipped with a nano-LC-based electrospray ionization source and coupled online to a Dionex Ultimate 3000 UPLC system (Thermo Fisher Scientific).

A “top 3 second” data-dependent tandem mass spectrometry approach was utilized to identify peptides in the samples. In the top 3 s scan protocol, a full scan spectrum (survey scan, 400–1650 Th) is acquired using an Orbitrap mass analyzer with 240,000 resolution followed by collision-induced dissociation mass spectra acquired in the linear ion trap of the most abundant ions in the survey scan for 3 s.

Raw MS files were processed using MaxQuant software54–56 (ver.2.0.3.0) against a database with forward and reversed Uniprot Human protein sequences, downloaded from www.uniprot.org on 1/19/2022. Standard settings in the MaxQuant were applied. Only proteins identified with a minimum of 2 unique peptides were considered. Peak areas (PAs) for each protein were obtained by selecting the label-free quantification (LFQ) option in MaxQuant. PP2Ac was detected in PP2Ac immunoprecipitates from all 64 samples. In addition, 2951 proteins were identified with minimum 2 unique peptides and with false discovery rates at 0.01 in the PP2Ac immunoprecipitates. To be considered as a PP2Ac interaction partner, a protein had to satisfy the following criteria, as described in Figure 2: (1) identified with LFQ_PA in > half of the PP2Ac IP (i.e., >32 out of 64 samples used); 934 proteins were identified in the same sample, which results in Norm:

This normalization strategy is widely used in proteomics studies involving protein–protein interactions39,46–49 and uses the same concept used in Western blotting, in which the Western blot signal for an interaction protein is normalized against that for the protein serving as the “bait”. The normalized PAs for each PP2Ac interaction partner, Norm:PA, for insulin-treated samples were divided by that for the corresponding sample without the insulin treatment (INS/INS) to assess the effect of insulin response. The resulting ratios (e.g., LN_INS/LN_BAS, OIR_INS/OIR_BAS, and so on) were log 2 transformed and compared across the 2 groups to determine effects of insulin resistance and Pio on PP2Ac interactions.

**Statistical Analysis.** Statistical significance was assessed using independent t-tests. Differences were considered statistically significant at *P < 0.05.

**Bioinformatics Analysis.** Pathway analysis on PP2Ac interaction partners were performed using database for annotation, visualization, and integrated discovery (David).57 A pathway was considered as significantly enriched if both the P-value for the pathway was less than 0.01 and the pathway included at least 4 of the identified PP2Ac partners. In addition, the largest phosphorylation site database was downloaded from eukaryotic phosphorylation sites database (EPSD) (biocuckoo.cn) to determine known phosphosites for each identified PP2Ac partners.

### RESULTS AND DISCUSSION

Sixteen human participants were included in this study. The clinical characteristics of these participants are listed in Table 1. No significant difference in age, fasting plasma glucose, and

| participant group | Lean | OIR |
|-------------------|------|-----|
| gender (M/F)      | (4/4)| (4/4)|
| age (years)       | 23.0±1.5 | 26.3±4.4 |
| BMI (kg/m²)       | 21.9±0.7 (<25) | 33.8±1.2 (>30)** |
| 2h OGTT glucose (mg/dl) | 93.4±6.8 (<140) | 125±3.2 (113 (<140)* |
| Hba1c (%)         | 5.3±0.1 (<5.7) | 5.6±0.1 (<5.7) |
| fasting plasma glucose (mg/dl) | 87.7±1.5 (<100) | 91.3±3.4 (100) |
| M-value (mg/kg/min) | 12.0±1.2 | 3.4±0.4** |

*Data are shown as mean ± SEM, n = 8. All measurements were done on overnight fasting. In parenthesis: Criteria for Lean/OIR, normal ranges for glucose tolerance, Hba1c, and fasting plasma glucose. Insulin-stimulated glucose disposal rates (M-value) were calculated as the average value during the last 30 min of insulin infusion. **P < 0.01 compared with lean. *P < 0.05 compared with lean.

Hba1c between the two groups. However, BMI and 2h OGTT glucose were significantly higher in OIR compared to Lean. In addition, the M-value, a measure for insulin sensitivity obtained during the hyperinsulinemic-euglycemic clamp, is significantly lower in OIR compared to Lean.

PP2Acα/β were detected in PP2Ac immunoprecipitates from all 64 samples used for the study. In total, 216 proteins met the criteria for classification as endogenous PP2Ac interaction partners, these proteins are listed in Table S1. Among those, 32 were reported as PP2Ac interaction partners, these proteins are listed in Table S1.
regulatory subunit A alpha isoform (PPP2R1A), 55 kDa regulatory subunit B subunit alpha (PPP2R3A), and 56 kDa regulatory subunit delta isoform (PPP2R5D). Therefore, 184 PP2Ac

Figure 3. Protein interaction network in HSKM. Twenty interactors with confidence larger than 0.9 were employed to compose the interaction network of PPP2CB. Proteins identified in this study are highlighted in red circles.

Figure 4. Example significantly enriched pathways for the 216 PP2Ac interaction partners and PP2Ac in HSKM revealed by proteomics and KEGG/REACTOME pathway analysis. The greater the $-\log_{10}(P\text{-value})$, the smaller $P$-value, and less likely a pathway is significantly enriched just by chance.
interaction partners identified in the current study appear to be novel. Furthermore, none of these 216 PP2Ac partners were previously identified as a PP2Ac partner in primary HSKM cells. These novel PP2Ac partners may help better understand the role and regulation of PP2A in various cellular processes in the skeletal muscle in humans. Note that these 216 partners may interact with PP2Ac directly or indirectly.

We performed network analysis on the determined PP2Ac partners using STRING database for the 216 PP2Ac interaction partners identified in this study. One network is shown in Figure 3 to demonstrate how some of these partners are interrelated.

Pathway analysis of the identified 216 interaction partners as well as PP2Ac were performed using DAVID compared to the whole genome background. The analysis showed various pathways are significantly enriched, such as mRNA surveillance whole genome background. The analysis showed various pathways influence the PP2A function.

Each of the 216 PP2Ac interaction partners has at least one phosphorylation site listed in largest phosphorylation database, EPSPD. In total, 10,960 human phosphorylation sites were assigned to these 216 PP2Ac interaction partners in the phosphorylation database (Table S2).

Our analysis has revealed that were 26 interaction partners with a higher insulin response in HSKM cells derived from OIR participants compared to lean subjects (Table 2). Figure 5 demonstrates the volcano plot for PP2Ac interaction partners in OIR_INS/OIR_BAS versus LN_INS/LN_BAS. Figure 6 provides a heat map for the 26 abnormal PP2Ac partners in OIR insulin versus basal (P < 0.05) with corresponding changes in the Pio treated conditions.

We analyzed these 26 PP2Ac partners using DAVID to determine their involved cellular pathways, molecular functions, and their domains features. Figures 7 and 8 show the significantly enriched pathways and molecular functions, respectively, such as nonsense-mediated decay, metabolism of RNA, RNA binding, and protein binding for the 26 PP2Ac interaction partners with a higher response to insulin in OIR versus Lean. The significant enriched domains for the 26 PP2Ac interaction partners with a higher response to insulin in OIR versus Lean included coiled coil domain, translation protein SH3-like domain, and ribosomal protein L2 domain 2. PP2R2A is a reported regulatory subunit of PP2Ac, which may modulate PP2Ac activity. Knockdown of PP2R2A

### Table 2. 26 PP2Ac Interaction Partners with Significant Higher Response to Insulin in OIR than in Lean with Corresponding Changes in the Pio-Treated Conditions

| gene name | protein name | LN_INS/ LN_BAS | OIR_INS/ OIR_BAS | OIR_Pio_INS/ OIR_Pio_BAS |
|-----------|--------------|----------------|------------------|-------------------------|
| CAMK2G    | calcium/calmodulin-dependent protein kinase II subunit gamma | 1.00 ± 0.08 | 1.60 ± 0.28* | 1.82 ± 0.24* |
| CDC42BPB  | serine/threonine-protein kinase MRCK beta | 1.00 ± 0.20 | 2.47 ± 0.89* | 2.64 ± 0.45* |
| CAMS      | N-acetylneuraminic cytidiyltransferase | 1.00 ± 0.11 | 1.62 ± 0.18* | 1.85 ± 0.31* |
| EIF4H     | eukaryotic translation initiation factor 4H | 1.00 ± 0.11 | 1.53 ± 0.13* | 1.56 ± 0.18* |
| EIF5A     | eukaryotic translation initiation factor 5A | 1.00 ± 0.10 | 1.99 ± 0.34* | 1.36 ± 0.19 |
| FOCD      | focal adhesion | 1.00 ± 0.17 | 2.09 ± 0.37* | 1.50 ± 0.07* |
| HSPA14    | heat shock 70 kDa protein 14 | 1.00 ± 0.12 | 3.29 ± 1.78* | 1.75 ± 0.43 |
| KIF1B     | kinesin-like protein KIF1B | 1.00 ± 0.12 | 1.74 ± 0.26* | 1.84 ± 0.31* |
| LUC7L2    | putative RNA-binding protein Luc7-like 2 | 1.00 ± 0.15 | 1.74 ± 0.33* | 2.68 ± 1.14 |
| MYL12A    | myosin regulatory light chain 12A | 1.00 ± 0.17 | 2.09 ± 0.34* | 2.02 ± 0.61 |
| NUFIP2    | nuclear fragile X mental retardation-interacting protein 2 | 1.00 ± 0.13 | 2.11 ± 0.41* | 1.32 ± 0.11 |
| NUMBL     | numb-like protein | 1.00 ± 0.13 | 1.59 ± 0.10* | 1.96 ± 0.25* |
| PDA1      | 28 kDa heat- and acid-stable phosphoprotein | 1.00 ± 0.17 | 2.25 ± 0.42* | 2.02 ± 0.28* |
| PELO      | protein pelota homologue | 1.00 ± 0.09 | 1.78 ± 0.20* | 1.86 ± 0.39* |
| PPP1R21   | protein phosphatase 1 regulatory subunit 21 | 1.00 ± 0.13 | 2.53 ± 0.23* | 1.85 ± 0.46 |
| PPP2R2A   | Serine/threonine-PP2A55 kD regulatory subunit B alpha isofrm | 1.00 ± 0.08 | 2.03 ± 0.27* | 1.51 ± 0.22* |
| PRRC2A    | protein PRRC2A | 1.00 ± 0.11 | 1.63 ± 0.25* | 1.78 ± 0.18* |
| RBM3      | RNA-binding protein 3 | 1.00 ± 0.09 | 1.51 ± 0.10* | 1.36 ± 0.15 |
| RPL14     | 60S ribosomal protein L14 | 1.00 ± 0.12 | 1.93 ± 0.34* | 1.66 ± 0.20* |
| RPL34     | 60S ribosomal protein L34 | 1.00 ± 0.15 | 1.93 ± 0.29* | 2.06 ± 0.36* |
| SMG8      | protein SMG8 | 1.00 ± 0.19 | 2.22 ± 0.45* | 2.98 ± 1.62 |
| TRIP10    | Cdc42-interacting protein 4 | 1.00 ± 0.11 | 1.68 ± 0.24* | 2.41 ± 1.22 |
| TRMT1L    | TRMT1-like protein | 1.00 ± 0.09 | 2.00 ± 0.40* | 1.96 ± 0.28* |
| VAC14     | protein VAC14 homologue | 1.00 ± 0.13 | 2.07 ± 0.22* | 2.07 ± 0.24* |
| VPS53     | vacuolar protein sorting-associated protein 53 homologue | 1.00 ± 0.14 | 1.70 ± 0.18* | 1.46 ± 0.19 |
| XRNR2     | 5–3 exoribonuclease 2 | 1.00 ± 0.08 | 2.29 ± 0.50* | 1.42 ± 0.17* |

*Data are given as fold changes (means ± SEM). PA for each protein identified in a specific sample was normalized against the PA for PP2Ac identified in the same sample (see the Experimental Section). The normalized PA for each PP2Ac interaction partner was compared within the group to assess effects of insulin and Pio or across the 2 groups to determine effects of insulin resistance on protein–protein interactions involving PP2Ac. Mean of the normalized PA for each PP2AC interaction partner in the lean basal condition was set to 1.00, and all the fold changes were relative to the lean basal. A fold change of 2 indicates a 2-fold increase, and a fold change of 0.5 indicates a 2-fold decrease. *, P < 0.05 compared to lean basal.
reduced β-cell death under oxidative stress in INS-1 cells, and protein abundance of PPP2R2A was higher in db/db T2D mouse islets. Our finding that PPP2R2A has higher insulin response in HSKM cells from OIR than Lean also suggests a role of PPP2R2A in the development of insulin resistance.

Cdc42-interacting protein 4 (aka TRIP10) is involved in insulin signaling and there is an interaction between TRIP-Br1, which is regulated by PP2A in cells. In this study, we provide the first evidence that the interaction between TRIP10 and PP2Ac had a higher insulin response in HSKM cells from OIR compared to Lean.

Multiple modulators of protein synthesis also had a higher response to insulin with regard to their association with PP2Ac in OIR versus Lean, such as eukaryotic translation initiation factor 4H (EIF4H), eukaryotic translation initiation factor 5A (EIF5A), 60S ribosomal protein L14 (RPL14), and 60S ribosomal protein L34 (RPL34). Eukaryotic translation initiation is established as an important process in protein synthesis. Generally, EIF4H activity involves directing other cellular proteins to specific mRNA structures to enhance their activity. Protein synthesis is reduced in T2D compared to control subjects, and the underlying mechanisms are unclear. Our current findings identified a potentially new mechanism for PP2A’s role in reduced protein synthesis in insulin resistance.

Proteins involved in maintaining cytoskeleton dynamics and muscle contraction play a major role in regulating glucose uptake and impairment in these processes may contribute to the development of insulin resistance. These proteins including calcium/calmodulin-dependent protein kinase type II subunit gamma (CAMK2G), serine/threonine-protein kinase MRCK beta (CDC42BPB), kinesin-like protein KIF1B (KIF1B), and myosin regulatory light chain 12A (MYL12A). CAMK2G is a member of a class of Ca (2+)/calmodulin (CaM)-dependent protein kinases and is a potent stimulator of Ca (2+)-dependent gene expression. It is being activated by phosphorylation on T200 by CaMKK. Interaction of CAMK2G with PP2Ac is reported previously and it negatively regulates CAMK2G function. CAMK2G/PP2Ac complex also binds IQGAP1, which has actin-binding activity and is regulated by calcium binding proteins (e.g., calmodulin). CDC42BPB is a serine/threonine-protein kinase which acts as a downstream effector of CDC42 and plays a role in the regulation of cytoskeleton reorganization and cell migration and regulates assembly and organization of actin cytoskeletal via phosphorylation of PPP1R12C and MYL9/MLC2. In addition, kinesin-like protein KIF1B (KIF1B) and myosin regulatory light chain 12A (MYL12A) also showed higher insulin-stimulated interaction with PP2Ac in cells derived from OIR compared to Lean, which might contribute

Figure 5. Volcano plot for PP2Ac interaction partners in response to insulin, OIR_INS/OIR_BAS vs LN_INS/LN_BAS.

Figure 6. Heat map for the 26 abnormal PP2Ac partners in OIR insulin vs basal (P < 0.05) with corresponding changes in the Pio treated conditions.
to the impairment in cytoskeleton dynamics and muscle contraction in insulin resistance.

We have looked at the effect of Pio on the abnormal partners of PP2Ac with higher association with PP2Ac in OIR upon insulin stimulation compared to the Lean group. Three PP2Ac partners (HSPA14, EIF5A, and NUFIP2) had >1.4 fold decrease (i.e., fold change <0.7) in their response to insulin in OIR after the Pio treatment (i.e., OIR_Pio_INS/OIR_Pio_BAS vs OIR_INS/OIR_BAS) and had no significant differences in their response to insulin in OIR after the Pio treatment versus Lean (i.e., \( P > 0.05 \) OIR_Pio_INS/OIR_Pio_BAS vs LN_INS/LN_BAS). These results suggested that Pio reversed the abnormality in these three interactions with PP2Ac in OIR and rendered them similar to Lean, which are shown in Figure 9.

The heat shock proteins (HSPs) are stress-responsive proteins. HSPA14 is member 14 of the HSP70 family. This family of proteins functions as chaperones, regulating the folding of other proteins and preventing aggregation and facilitate degradation of misfolded proteins. It has a crucial role in diabetes because uncontrolled oxidative stress represents a characteristic feature of diabetes. HSPA14 is involved in several processes, including a cellular response to unfolded proteins: binding to misfolded proteins as well as a negative role in insulin signaling.

Modifications of post-translational mechanisms of these proteins may affect its function and may contribute to diabetic complications. This is possibly in agreement with our findings where in insulin resistance the association with PP2Ac was higher, which implies that phosphorylation of HSPA14 may be less and may reduce its antioxidant activity. On the other hand, Pio decreased the interaction with PP2Ac and normalized it to lean level, which might enhance insulin sensitivity.
Inflammation and insulin resistance are highly correlated. Eukaryotic translation initiation factor 5A (eIF5A) is a mediator of cellular proliferation and apoptosis and is essential to inflammatory responses. It was found that the depletion of eIF5A and the inhibition of its hypusination in diabetic mice led to resistance to β-cell loss and hyperglycemia development. It was reported that eIF5A interacts with multiple different proteins. Our results showed that there is higher association of eIF5A with PP2Ac in the OIR group after insulin stimulation, which suggests that PP2Ac may play a role in inflammation-mediated insulin resistance. Interestingly, Pio was able to normalize this interaction to levels similar to the lean group, which might contribute to Pio’s insulin-sensitizing effect.

Proteomic approaches using Co-IP have been widely used to study protein–protein interaction networks, however, one limitation of Co-IP exits: the proteins bound to the bait (in this case, PP2Ac) may have different binding in different cell types. Moreover, in the current study, multiple reported PP2Ac interaction partners were not identified as PP2Ac interaction partners in HSKM cells, such as IRS1, AMPK, AKT, and so on. One possible explanation is the low abundance of these proteins in HSKM cells. In addition, experiments in cell culture and/or animal models might not translate into humans. Therefore, muscle tissue samples from human participants, which better reflect human physiology/pathophysiology, are needed to confirm the in-vitro study results.

**CONCLUSIONS**

Protein–protein interactions are important in regulating various signaling pathways including insulin signaling. Abnormalities in these processes are the main cause in the development of skeletal muscle insulin resistance. This study reports the largest experimentally determined PP2Ac interaction partners in Pio and/or insulin-treated HSKM cells derived from lean insulin-sensitive and OIR participants, with 216 identified PP2Ac partners (most of them are novel). We also discovered novel abnormal insulin-responsive PP2Ac partners in OIR groups. Furthermore, we revealed that Pio reversed the abnormality in some PP2Ac interaction partners in OIR and rendered them similar to Lean. These results provide a molecular framework for a better understanding on how protein complexes of PP2Ac differ in Lean and OIR and how Pio may restore certain abnormal PP2Ac protein–protein interactions. These results may help understand the molecular mechanisms underlying insulin resistance and the insulin sensitizing effects of Pio treatments, providing multiple targets from different signaling pathways to reverse insulin resistance and prevent and/or manage T2D with less drugs side effects.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c04473.

216 PP2Ac partners identified by Co-IP and UPLC-ESI-MS/MS in the study and known phosphorylation sites for the 216 PP2Ac partners identified in the study (XLSX)

**AUTHOR INFORMATION**

**Corresponding Author**

Zhengping Yi – Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Wayne State University, Detroit, Michigan 48201, United States; orcid.org/0000-0002-2975-7205; Phone: (313) 577-0823; Email: zhengping.yi@wayne.edu

**Authors**

Lana Alghanem – Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Wayne State University, Detroit, Michigan 48201, United States

Xiangmin Zhang – Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Wayne State University, Detroit, Michigan 48201, United States

Ruchi Jaiswal – Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, Wayne State University, Detroit, Michigan 48201, United States

Berhane Seyoum – Division of Endocrinology, Wayne State University School of Medicine, Wayne State University, Detroit, Michigan 48201, United States

Abdullah Mallisho – Division of Endocrinology, Wayne State University School of Medicine, Wayne State University, Detroit, Michigan 48201, United States

Zaher Msallaty – Division of Endocrinology, Wayne State University School of Medicine, Wayne State University, Detroit, Michigan 48201, United States

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c04473

**Author Contributions**

All authors contributed equally to this work.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by NIH/NIDDK, R01 DK107666 (Z.Y.), R01 DK081750 (Z.Y.), and R01 DK128937 (Z.Y.), American Diabetes Association Translational Science award #1-13-TS-27 & Clinical/Translational award #7-09-CT-56 (Z.Y.), Wayne State University Diabetes and Obesity Team Science, bridge funding, and grant boost (Z.Y.). No potential conflicts of interest relevant to this article were reported. L.A. designed and performed clinical and proteomic experiments, analyzed data, generated figures, and wrote the manuscript. X.Z. helped with proteomic experiments, analyzed data, generated figures, and wrote the manuscript. B.S., A.M., and Z.M. carried out muscle biopsy and hyperinsulinemic-euglycemic clamp experiments and performed other clinical related tasks. R.J. helped with proteomic experiments. Z.Y. is the guarantor of this work and, as such, supervised the project, designed the clinical and proteomic experiments, analyzed data, generated figures, helped with data interpretation, and wrote the manuscript. We thank study participants, and the staff at the clinical research center and other research assistants for carrying out clinical study-related tasks.

**REFERENCES**

(1) International Diabetes Federation. IDF Diabetes Atlas 2021. 2021https://diabetesatlas.org/ (accessed on 07/01/2022).

(2) Centers for Disease Control and Prevention. National Diabetes Statistics Report. 2022https://www.cdc.gov/diabetes/data/statistics-report/index.html (accessed on 07/01/2022).
(3) Abdul-Ghani, M. A.; DeFronzo, R. A. Pathogenesis of insulin resistance in skeletal muscle. J. Biomed. Biotechnol. 2010, 2010, 476279.

(4) Petersen, M. C.; Shulman, G. I. Mechanisms of Insulin Action and Insulin Resistance. Physiol. Rev. 2018, 98, 2133–2223.

(5) Feraco, A.; Gorini, S.; Armani, A.; Camajani, E.; Rizzo, M.; Caprio, M. Exploring the Role of Skeletal Muscle in Insulin Resistance: Lessons from Cultured Cells to Animal Models. Int. J. Mol. Sci. 2021, 22, 9327.

(6) Lee, S. H.; Park, S. Y.; Choi, C. S. Insulin Resistance: From Mechanisms to Therapeutic Strategies. Diabetes Metab. J. 2022, 46, 15–37.

(7) Lei, W. S.; Kindler, J. M. Insulin resistance and skeletal health. Curr. Opin. Endocrinol. Diabetes Obes. 2022, 29, 343–349.

(8) Merz, K. E.; Thurmond, D. C. Role of Skeletal Muscle in Insulin Resistance and Glucose Uptake. Compr. Physiol. 2020, 10, 785–809.

(9) Frangos, S. M.; Bishop, D. J.; Holloway, G. P. Revisiting the contribution of mitochondrial biology to the pathophysiology of skeletal muscle insulin resistance. Biochem. J. 2021, 478, 3809–3826.

(10) Omta, U.; Araki, A. Skeletal muscle as a treatment target for older adults with diabetes mellitus: The importance of a multimodal intervention based on functional category. Geriatr. Gerontol. Int. 2022, 22, 110–120.

(11) Barrett, J. S.; Whytock, K. L.; Strauss, J. A.; Wagenmakers, A. J. M.; Shepherd, S. O. High intramuscular triglyceride turnover rates and the link to insulin sensitivity: influence of obesity, type 2 diabetes and physical activity. Appl. Physiol., Nutr., Metab. 2022, 47, 343–356.

(12) White, M. F.; Kahn, C. R. Insulin action at a molecular level - 100 years of progress. Mol. Metab. 2021, 52, 101304.

(13) DeFronzo, R. A.; Tripathy, D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care 2009, 32, S157–S163.

(14) Abdul-Ghani, M. A.; DeFronzo, R. A. Pathogenesis of insulin resistance in skeletal muscle. J. Biomed. Biotechnol. 2010, 2010, 476279.

(15) Samuel, V. T.; Shulman, G. I. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. J. Clin. Invest. 2016, 126, 12–22.

(16) Haider, N.; Lebastchi, J.; Jayavelu, A. K.; Batista, T. M.; Pan, H.; Dreyfuss, J. M.; Carcamo-Orive, I.; Knowles, J. W.; Mann, M.; Kahn, C. R. Signaling defects associated with insulin resistance in nondiabetic and diabetic individuals and modification by sex. J. Clin. Invest. 2021, 131, No. e151818.

(17) Martins, A. R.; Nachbar, R. T.; Gorjao, R.; Vinolo, M. A.; Festuccia, W. T.; Lamberti, R. H.; Cury-Boaventura, M. F.; Silveira, L. R.; Curi, R.; Hirabara, S. M. Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. Lipids Health Dis. 2012, 11, 30.

(18) Di Meo, S.; Iossa, S.; Venditti, P. Skeletal muscle insulin resistance: role of mitochondria and other ROS sources. J. Endocrinol. 2017, 233, R15–R42.

(19) Luo, W.; Ai, L.; Wang, B. F.; Zhou, Y. High glucose inhibits myogenesis and induces insulin resistance by down-regulating AKT signaling. Biomed. Pharmacothero. 2019, 120, 109498.

(20) Liu, Z.; Zhu, H.; He, C.; He, T.; Pan, S.; Zhao, N.; Zhu, L.; Guan, G.; Liu, P.; Zhang, Y.; Wang, J. Nicorandil attenuates high glucose-induced insulin resistance by suppressing oxidative stress-mediated ER stress PERK signaling pathway. BMJ Open Diabetes Res. Care 2021, 9, No. e001884.

(21) Lu, P.; Zhao, Z. Advances on PPARgamma Research in the Emerging Era of Precision Medicine. Curr. Drug Targets 2018, 19, 663–673.

(22) Tyagi, S.; Sharma, P.; Gupta, A. S.; Saini, C.; Kaushal, S. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. J. Adv. Pharm. Technol. Res. 2011, 2, 236–240.

(23) Schernthaner, G.; Matthews, D. R.; Charbonnel, B.; Hanefeld, M.; Brunetti, P.; Quartet Study, G. Efficacy and safety of pioglitazone versus metformin in patients with type 2 diabetes mellitus: a double-blind, randomized trial. J. Clin. Endocrinol. Metab. 2004, 89, 6068–6076.

(24) Verma, N. K.; Singh, J.; Dey, C. S. PPAR-gamma expression modulates insulin sensitivity in C2C12 skeletal muscle cells. Br. J. Pharmacol. 2004, 143, 1006–1013.

(25) Bays, H.; Mandarino, L.; DeFronzo, R. A. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxisomal proliferator-activated receptor agonists provide a rational therapeutic approach. J. Clin. Endocrinol. Metab. 2004, 89, 463–478.
(41) Gu, P.; Qi, X.; Zhou, Y.; Wang, Y.; Gao, X. Generation of Ppp2Ca and Ppp2Cb conditional null alleles in mouse. *Genesis* 2012, 50, 429–436.

(42) Götz, J.; Probst, A.; Ehler, E.; Hemmings, B.; Kues, W. Delayed embryonic lethality in mice lacking protein phosphatase 2A catalytic subunit Calpha. *Proc. Natl. Acad. Sci. U.S.A.* 1998, 95, 12370–12375.

(43) Pan, X.; Chen, X.; Tong, X.; Tang, C.; Li, J. Ppp2ca knockout in mice spermatoogenesis. *Reproduction* 2015, 149, 385–391.

(44) Xian, L.; Hou, S.; Huang, Z.; Tang, A.; Shi, P.; Wang, Q.; Song, A.; Jiang, S.; Lin, Z.; Guo, S.; Gao, X. Liver-specific deletion of Ppp2calpha enhances glucose metabolism and insulin sensitivity. *Aging* 2015, 7, 223–232.

(45) Amin, F.; Awal, S.; Vigneron, S.; Roque, S.; Mechali, F.; Labbé, J. C.; Lovca, T.; Castro, A. PPA2-B55: substrates and regulators in the control of cellular functions. *Oncogene* 2022, 41, 1–14.

(46) Marcilla, M.; Albar, J. P. Quantitative proteomics: A strategic ally to map protein interaction networks. *IUBMB Life* 2013, 65, 9–16.

(47) Zhang, X.; Damacharla, D.; Ma, D.; Qi, Y.; Tagett, R.; Draghi, S.; Kowluru, A.; Yi, Z. Quantitative proteomics reveals novel protein interaction partners of PP2A catalytic subunit in pancreatic beta-cells. *Mol. Cell. Endocrinol.* 2016, 424, 1–11.

(48) Geetha, T.; Langalis, P.; Luo, M.; Mapes, R.; Lefort, N.; Chen, S. C.; Mandarino, L. J.; Yi, Z. Label-free proteomic identification of endogenous, insulin-stimulated interaction partners of insulin receptor substrate-1. *J. Am. Soc. Mass Spectrom.* 2011, 22, 457–466.

(49) Caruso, M.; Zhang, X.; Ma, D.; Yang, Z.; Qi, Y.; Yi, Z. Novel Endogenous, Insulin-Stimulated Akt2 Protein Interaction Partners In L6 Myoblasts. *PLoS One* 2015, 10, No. e014255.

(50) Damacharla, D.; Thamilselvan, V.; Zhang, X.; Mestareehi, A.; Yi, Z.; Kowluru, A. Quantitative proteomics reveals novel interaction partners of Rac1 in pancreatic beta-cells: Evidence for increased interaction with Rac1 under hyperglycemic conditions. *Mol. Cell. Endocrinol.* 2019, 494, 110489.

(51) Mestareehi, A.; Zhang, X.; Seyoum, B.; Msallaty, Z.; Mallisher, A.; Burghardt, K. J.; Kowluru, A.; Yi, Z. Metformin Increases Protein Phosphatase 2A Activity in Primary Human Skeletal Muscle Cells Derived from Lean Healthy Participants. *J. Diabetes Res.* 2021, 2021, 9979234.

(52) Qi, Y.; Zhang, X.; Seyoum, B.; Msallaty, Z.; Mallisher, A.; Caruso, M.; Damacharla, D.; Ma, D.; Al-Janabi, W.; Tagett, R.; Alharbi, M.; Calme, G.; Mestareehi, A.; Draghi, S.; Abou-Samra, A.; Kowluru, A.; Yi, Z. Kinome Profiling Reveals Abnormal Activity of Kinases in Skeletal Muscle From Adults With Obesity and Insulin Resistance. *J. Clin. Endocrinol. Metab.* 2020, 105, 644.

(53) Burghardt, K. J.; Calme, G.; Caruso, M.; Howlett, B. H.; Sanders, E.; Msallaty, Z.; Mallisher, A.; Seyoum, B.; Qi, Y. A.; Zhang, X.; Yi, Z. Profiling the Skeletal Muscle Proteome in Patients on Atypical Antipsychotics and Mood Stabilizers. *Brain Sci.* 2022, 12, 259.

(54) de Godoy, L. M.; Olsen, J. V.; Cox, J.; Nielsen, M. L.; Hubner, N. C.; Fröhlich, F.; Walther, T. C.; Mann, M. Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. *Nature* 2008, 455, 1251–1254.

(55) Cox, J.; Mann, M. A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat. Protoc.* 2009, 4, 698–705.

(56) Neuhausser, N.; Michalski, A.; Cox, J.; Mann, M. Expert system for computer-assisted annotation of MS/MS spectra. *Mol. Cell. Proteomics* 2012, 11, 1500–1509.

(57) Thomas, S.; Bonchev, D. A survey of current software for network analysis in molecular biology. *Hum. Genomics* 2010, 4, 353–360.

(58) Shi, Y. Serine/threonine phosphatases: mechanism through structure. *Cell* 2009, 139, 468–484.

(59) Yan, L.; Guo, S.; Braut, M.; Harmon, J.; Robertson, R. P.; Hamid, R.; Stein, R.; Yang, E. The B55α-containing PP2A holoenzyme dephosphorylates FOXO1 in islet β-cells under oxidative stress. *Biochem. J.* 2012, 444, 239–247.

(60) Zang, Z. J.; Gunaratnam, L.; Cheong, J. K.; Lai, L. Y.; Hisao, L. L.; Oleary, E.; Sun, X.; Saltotellez, M.; Bonventre, J. V.; Hsu, S. L. Identification of PP2A as a novel interactor and regulator of TRIP-Bri. *Cell. Signal.* 2009, 21, 34–42.

(61) Sun, Y.; Atas, E.; Lindqvist, L.; Sonenberg, N.; Pelletier, J.; Mellor, A. The eukaryotic initiation factor eIF4F facilitates loop-binding, repetitive RNA unwinding by the eIF4A DEAD-box helicase. *Nucleic Acids Res.* 2012, 40, 6199–6207.

(62) Halvatsiotis, P.; Short, K. R.; Bigelow, M.; Nair, K. S. Synthesis rate of muscle proteins, muscle functions, and amino acid kinetics in type 2 diabetes. *Diabetes* 2002, 51, 2395–2404.

(63) Hoffman, N. J.; Elmdorph, J. S. Signaling, cytoskeletal and membrane mechanisms regulating GLUT4 exocytosis. *Trends Endocrinol. Metab.* 2011, 22, 110–116.

(64) Stöckli, J.; Fazakerley, D. J.; James, D. E. GLUT4 exocytosis. *J. Cell Sci.* 2011, 124, 4417–4419.

(65) Anderson, K. A.; Noeldner, P. K.; Reeke, C.; Wadzinski, B. E.; Means, A. R. Regulation and function of the calcium/calmodulin-dependent protein kinase IV/protein serine/threonine phosphatase 2A signaling complex. *J. Biol. Chem.* 2004, 279, 31708–31716.

(66) Bashour, A. M.; Fullerton, A. T.; Hart, M. J.; Bloom, G. S. iQGAP1, a Rac- and Cdc42-binding protein, directly binds and cross-links microfilaments. *J. Cell Biol.* 1997, 137, 1555–1566.

(67) Sayed, K. M.; Mahmoud, A. A. Heat shock protein-70 and hypoxia inducible factor-1alpha in type 2 diabetes mellitus patients complicated with retinopathy. *Acta Ophthalmol.* 2016, 94, e361–e366.

(68) Singh, K.; Agrawal, N. K.; Gupta, S. K.; Mohan, G.; Chaturvedi, S.; Singh, K. Decreased expression of heat shock proteins may lead to compromised wound healing in type 2 diabetes mellitus patients. *J. Diabetes Its Complications* 2015, 29, 578–588.

(69) Atalay, M.; Oksala, N.; Lappalainen, J.; Laaksonen, D. E.; Sen, C. K.; Roy, S. Heat shock proteins in diabetes and wound healing. *Curr. Protein Pept. Sci.* 2009, 10, 85–95.

(70) Odeggaard, J. I.; Chawla, A. Pleiotropic Actions of Insulin Resistance and Inflammation in Metabolic Homeostasis. *Science* 2013, 339, 172–177.

(71) Dali-Youcef, N.; Meccia, M.; Ricci, R.; Andrès, E. Metabolic inflammation: Connecting obesity and insulin resistance. *Ann. Med.* 2013, 45, 242–253.

(72) Maier, B.; Oghara, T.; Trace, A. P.; Tersey, S. A.; Robbins, R. D.; Chakrabarti, S. K.; Nunemaker, C. S.; Stull, N. D.; Taylor, C. A.; Thompson, J. E.; Dondero, R. S.; Lewis, E. C.; Dinarello, C. A.; Nadler, J. L.; Mirrira, R. G. The unique hypusine modification of eIF5A promotes islet beta cell inflammation and dysfunction in mice. *J. Clin. Invest.* 2010, 120, 2156–2170.

(73) Sievert, H.; Venz, S.; Platas-Barradas, O.; Dhole, V. M.; Schaletsky, M.; Nagel, C. H.; Braig, M.; Preukschas, M.; Fällmann, N.; Bokemeyer, C.; Brümmendorf, T. H.; Pörtner, R.; Walthr, R.; Duncan, K. E.; Hauber, J.; Babanov, S. Protein-protein-interaction network organization of the hypusine modification system. *Mol. Cell. Proteomics* 2012, 11, 1289–1305.