



Dysregulation of PP2A-Akt interaction contributes to Sucrose non-fermenting related kinase (SNRK) deficiency induced insulin resistance in adipose tissue

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ABSTRACT

Objective: We previously identified Sucrose non-fermenting related kinase (SNRK) as a regulator of adipose inflammation and energy homeostasis. In this study, we aimed to investigate the role of SNRK in insulin signaling in white (WAT) and brown adipose tissue (BAT).

Methods: Adipose tissue specific (SNRK deficiency in both WAT and BAT) and BAT specific knockout mouse models were employed. Phosphoproteomic studies were conducted to identify the novel SNRK pathway regulating insulin signaling in adipose tissue.

Results: SNRK ablation is sufficient to inhibit insulin-stimulated AKT phosphorylation and glucose uptake in both WAT and BAT. Phosphoproteomic study using SNRK deficient versus wild type BAT samples revealed 99% reduction of phosphorylation on Serine 80 of PPP2R5D, the regulatory subunit of Protein phosphatase 2A (PP2A). Drastic (142.5-fold) induction of phosphorylation on Serine 80 of PPP2R5D was observed in SNRK-deficient primary brown adipocytes overexpressing SNRK compared to control protein. *In vitro* phosphorylation reaction followed by targeted phosphoproteomic detection further confirms that human recombinant SNRK is able to phosphorylate human recombinant PPP2R5D. Dephosphorylated PPP2R5D promotes constitutive assembly of PP2A-AKT complex, therefore inhibits insulin-induced AKT phosphorylation and subsequent glucose uptake in both BAT and WAT. Knockdown of PPP2R5D in adipocytes can improve insulin sensitivity in adipocytes without SNRK expression.

Conclusions: Our findings demonstrate that SNRK regulates insulin signaling through controlling PPP2R5D phosphorylation, which subsequently impacts PP2A activity and then AKT phosphorylation in both WAT and BAT. SNRK may represent a promising potential target for treating insulin resistance-related metabolic disorders.

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Keywords Adipose tissue; SNRK; Insulin signaling; AKT phosphorylation; Phosphoproteomics

1. INTRODUCTION

Resistance to insulin-stimulated glucose uptake has been proven to play a central role in a cluster of metabolic diseases, including type 2 diabetes, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular diseases [1]. However, specific molecular mechanisms underlying insulin resistance remains incompletely understood. Extensive investigations including ours demonstrate that macrophage-related inflammatory activities in white adipose tissue (WAT) is considered as a causal factor for the development of obesity-related insulin resistance [2,3]. Recent identification of functional brown adipose tissue (BAT) in adult humans rekindled the hope of searching for BAT harnessing strategies for regulating glucose homeostasis [4]. BAT transplantation in mice [5] and activation of BAT by cold exposure in

humans [6] have shown favorable effects on glucose homeostasis and insulin sensitivity, albeit the underlying mechanisms remain unknown. As a critical regulator for carbohydrate metabolism in plants, SNRK has been studied extensively [7]. However, there have been limited investigations regarding the role of SNRK in mammalian cells and animal models. SNRK is expressed abundantly in both WAT and BAT [8]. We have previously identified SNRK as a novel regulator of adipose inflammation and energy homeostasis in both mice and humans [8,9]. In addition, SNRK has been reported to be essential for cardiac metabolism [10–12], angiogenesis [13], and angioblast development [14].

Based on the role of SNRK in carbohydrate metabolism in plant and our previous findings, we hypothesized that SNRK plays important roles in adipose glucose metabolism in mice. This study focuses on elucidating

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the role of SNRK in adipose glucose metabolism and insulin signaling and dissecting its differential roles in WAT versus BAT. The adipose tissue-specific (SNRK deficiency in both WAT and BAT) and BAT-specific (SNRK deficiency exclusively in BAT) SNRK KO models were used. SNRK ablation is sufficient to inhibit insulin-regulated AKT phosphorylation and glucose uptake in both BAT and WAT. Through applying global phosphoproteomic profiling and targeted phosphoproteomic assay, a novel SNRK pathway regulating insulin signaling in adipose tissue is identified.

2. MATERIALS AND METHODS

2.1. Reagents

Insulin, dexamethasone (Dex), isobutylmethylxanthine (IBMX), indomethacin (IDMT), and triiodothyronine (TIDT) were purchased from Sigma (St. Louis, MO); mouse anti-SNRK monoclonal antibody (sc-398557) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Akt (9272S), P-T308 Akt (9275S), P-S473 Akt (9271S), and PPP2R5D (5687S) antibodies were purchased from Cell Signaling Technology (Cambridge, MA), and protein phosphatase 2A (PP2A) Immunoprecipitation Phosphatase Assay Kit and anti-PP2A Antibody, C subunit were purchased from MilliporeSigma (Burlington, MA). Mouse lentiviral *Snrk* sgRNA, *Cas9* nuclease expression vectors, Accell mouse *Ppp2r5d* siRNA, and non-targeting siRNA were purchased from Dharmacon (Lafayette, CO); CAS9 antibody from Novus Biologicals (Centennial, CO), 2-[1,2-³H(N)]-Deoxy-D-glucose (³H-DG) was purchased from PerkinElmer (Waltham, MA). Recombinant human SNRK (active) and PPP2R5D proteins were purchased from Abcam (Cambridge, MA) and OriGene (Rockville, MD), respectively.

2.2. Cells

Primary brown preadipocytes were isolated from the brown adipose tissue of the global SNRK knockout and littermate wild-type (WT) pups 24 h within birth. These cells were immortalized through transformation with SV40 large T antigen as previously described [9,15]. For differentiation, brown preadipocytes were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and induced with 1 μg/ml insulin, 1 μM Dex, 0.5 mM IBMX, 0.125 mM IDMT, and 1 nM TIDT for 3 days. After induction, cells were maintained in DMEM containing 10% FBS and 1 μg/ml insulin for 5 additional days. 3T3-L1 cells were obtained from American Type Culture Collection and differentiated as previously described [16]. Insulin was removed from medium two days prior to experiments.

SNRK deficient 3T3-L1 (3T3-L1^{SNRK^{-/-}}) subline was generated through CRISPR-Cas9 technology using Dharmacon's Edit-R CRISPR-Cas9 system. 3T3-L1 cells were first transduced with the inducible lentiviral Cas9 nuclease expression vector followed by blasticidin selection for generation of a stable 3T3-L1 cell line carrying Cas9. Cells were then transduced with lentiviral sgRNA followed with puromycin selection. SNRK deficient 3T3-L1 cells were generated upon doxycycline induced Cas9 expression, which creates a frame shift in the SNRK gene and terminates protein transcription prematurely. DNA mismatches were confirmed by T7 Endonuclease I assay. Cloned 3T3-L1 cells were examined for SNRK gene mutations by Sanger sequencing using genomic PCR products around the target site. The cloned cells with mutation in the SNRK gene that caused a frame shift were used as SNRK knock out cells. The cloned cells without mutation in the SNRK gene were used as control cells.

2.3. Generation of tissue-specific SNRK-deficient mice

Details on the generation of the SNRK flox line (which have been made available at The Jackson Laboratory with the Stock No. 033222) were described in our previous publication [9]. In this study, SNRK^{loxP/loxP} mice were mated with adiponectin-Cre (A-Cre) transgenic mice to generate the adipose-specific SNRK-deficient mice (SNRK^{-/-,A-Cre}), and with UCP-1-Cre (U-Cre) transgenic mice to generate the brown adipose-specific SNRK-deficient mice (SNRK^{-/-,U-Cre}). The A-Cre and U-Cre lines were maintained by mating A-Cre or U-Cre mice with wild type mice. SNRK^{loxP/loxP}, SNRK^{A-Cre}, and SNRK^{U-Cre} littermates were used as controls.

2.4. Insulin signaling and glucose uptake assay

For the *in vivo* insulin signaling and glucose uptake experiment, mice were fasted overnight. A mixture of insulin (0.5 U/kg) and Deoxy-D-glucose, 2-[1,2-³H (N)] (³H-DG, 130 uCi/kg) was injected through portal vein under anesthesia. Epididymal adipose tissue and interscapular brown adipose tissue were dissected ten minutes after injection and immediately frozen in liquid nitrogen. Adipose tissues were homogenized in lysis buffer to generate protein lysates and Akt phosphorylation at T308 and S473 was detected using western blot analysis. To determine adipose tissue glucose uptake, adipose tissues were digested with 0.5 M NaOH overnight. Digested protein lysates were mixed with scintillation cocktail at the ratio of 1 mg versus 5 ml ³H-DG signals were measured with a liquid scintillation counter (B281000, PerkinElmer, IL). Details on glucose (GTT) and insulin (ITT) tolerance tests have been described elsewhere [9].

For *in vitro* insulin signaling, fully differentiated 3T3-L1 and primary brown adipocytes (PBAs) were incubated in serum-free DMEM overnight and then incubated in serum-free DMEM supplemented with PBS or various concentrations of insulin for 10 min, 30 min, 1 h, and 2 h at 37 °C. Cells were lysed in lysis buffer. AKT phosphorylation at T308 and S473 was detected using western blot.

For *in vitro* glucose uptake assay, the fully differentiated adipocytes were starved in serum-free DMEM overnight. Cells were washed once with freshly made KRH buffer (1X KRP/20 mM Hepes/1%BSA) and then incubated in 1 ml KRH buffer with various concentrations of insulin for 10 min at 37 °C. Then [³H]-DG was added to cells at a final concentration of 1 μCi/ml for additional 10-minute incubation at 37 °C. 50uM cytochalasin B was used to determine the nonspecific binding. After the incubation, cells were washed with cold KRH buffer three times, and lysed in 0.1% SDS for counting radioactivity.

2.5. Phosphoproteomic studies

To identify potential SNRK substrates in BAT, two global phosphoproteomic studies were conducted. The first one was to compare changes of phospho signals between WT and SNRK deficient brown adipose tissue. The second one was to re-introduce SNRK to SNRK deficient PBAs (PBA^{SNRK^{-/-}}) and examine for changes of phospho signals. PBA^{SNRK^{-/-}} were infected with retrovirus to express a truncated adenovirus receptor, followed by infection with adenoviruses expressing mouse SNRK (AdSNRK) or GFP (AdGFP). Protein lysates were extracted from BAT and PBA using freshly made 9M urea lysis buffer. Cell lysate was treated as previously described for phosphoproteomic study [8]. The log-transformed data were analyzed using two-tailed *t* test. To account for potential false positives because of the multiple comparisons in this study, the false discovery rate (FDR) was calculated by incorporating all *P* values from multiple tests. The FDR statistics with *q* ≤ 0.05 were considered significant [17].

To validate if PPP2R5D is the direct substrate of SNRK, an *in vitro* phosphoproteomic assay was conducted. In brief, the purified recombinant human SNRK (active) and PPP2R5D proteins were added in a test tube containing reaction buffer (50 mM Tris-HCl [pH 7.5], 0.1 mM EGTA, 0.1% v/v 2-mercaptoethanol, 1 mM ATP, 10 mM Mg acetate) for *in vitro* phosphorylation reaction. After 30-min incubation at 37 °C, the reaction was stopped by EDTA. The reaction products were then shipped on dry ice to the Science Exchange Proteomics and Mass Spectrometry Facility for targeted phosphoproteomic analysis.

2.6. PP2A activity assay

PP2A activity was detected using a PP2A immunoprecipitation phosphatase assay kit (MilliporeSigma, MA). Briefly, adipose tissues and adipocytes were lysed using phosphatase extraction buffer. 200 µg protein lysate was precleared with 30 µl protein A agarose beads for 30 min at 4 °C and then was incubated with 2 µg of PP2A antibody at 4 °C for 1 h. Then 30 µl protein A beads were added and incubated at 4 °C overnight. Beads were washed with cold TBS for 3 times and assay buffer once. 60 µl 1 mM phosphopeptide solution and 20 µl assay buffer were added to the dried beads followed by 10 min incubation at 30 °C. After a brief centrifugation, 25 µl supernatant was transferred into the well of microtiter plate. 100 µl of Malachite Green Phosphatase Detection Solution was added to develop color for 10 min at room temperature, followed by absorbance at 650 nm.

2.7. PPP2R5D siRNA knockdown

Dharmacon Accell siRNA was used to knockdown *Ppp2r5d* in adipocytes. The differentiated adipocytes were treated with 3 µM Accell *Ppp2r5d* or green non-targeting siRNA at 37 °C for 8 days. The *Ppp2r5d* mRNA knockdown was assessed using real-time PCR. Details on RNA extraction and Real-time PCR analysis have been described elsewhere [9]. Primer sequences are listed in Supplementary Table 1.

2.8. Co-Immunoprecipitations and western blotting analysis

To immunoprecipitate endogenous AKT, cell or tissue lysates were precleared with 30 µl of TrueBlot anti-Rabbit Ig IP Agarose beads (Rockland, PA), followed by incubation with AKT antibody for 1 h at 4 °C. Immunoprecipitation was performed with 30 µl IP beads overnight at 4 °C, immunoprecipitated complex were washed 4 times with lysis buffer and boiled in protein loading buffer. After SDS-PAGE, proteins were transferred onto PVDF membranes. Membranes were blocked in 5% milk/1 x TBST at RT for 1 h, then incubated with primary antibodies at 4 °C overnight, followed by incubation with HRP-linked anti-rabbit IgG (TrueBlot, Rockland) for 1 h at RT. Protein bands were detected by ECL western blotting detection reagent (PerkinElmer, MA).

2.9. Statistical analysis

Data are presented as the mean and SEM. Student *t* test was used to compare the difference between two groups. One-way ANOVA followed with Bonferroni *post hoc* test was employed when the groups are greater than two. Two-way or three-way ANOVA followed with Bonferroni *post hoc* test was used in the case of multiple treatments. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. SNRK is essential for insulin-regulated AKT phosphorylation and glucose uptake in adipose tissue

To dissect the role of SNRK in WAT versus BAT in insulin signaling and glucose homeostasis, two tissue-specific SNRK knockout mouse

models were generated, the adipose tissue-specific knockout model (SNRK^{-/-,A-Cre}) as previously reported [9] and the BAT-specific SNRK knockout model (SNRK^{-/-,U-Cre}). SNRK ablation in both WAT and BAT simultaneously significantly increased body weight and caused systemic inflammation (Figure 1A and Reference [9]). Interestingly, the systemic inflammation exclusively originated from the WAT of SNRK^{-/-,A-Cre} mice, because expression of cytokine and chemokine genes were unchanged in BAT of these mice (Figure 1B). SNRK deficiency in BAT alone did not affect body weight, glucose and insulin tolerance, or inflammatory events (Figure 1C–F).

Nevertheless, the glucose uptake capability is significantly blunted in primary brown adipocytes lacking SNRK (Figure 2A). This could be due to impaired insulin signaling since insulin stimulated AKT phosphorylation is attenuated on both threonine 308 (T308) and serine 473 (S473) (Figure 2B). Similar results were also observed in 3T3-L1 adipocytes with SNRK deleted by CRISPR technology, which showed impaired glucose uptake and decreased insulin signaling (Figure 2C–D). *In vivo* experiments were performed to examine whether SNRK deficiency in adipose tissue impairs glucose uptake and insulin signaling. As shown in Figure 2E, the ability of glucose uptake in BAT was eliminated in SNRK^{-/-,U-Cre} mice compared to littermate control SNRK^{loxP/loxP} and SNRK^{U-Cre} mice. Insulin-stimulated AKT phosphorylation is also blunted in BAT of SNRK^{-/-,U-Cre} mice (Figure 2F). In SNRK^{-/-,A-Cre} mice, glucose uptake and insulin signaling were impaired in both WAT and BAT in comparison to littermate control SNRK^{loxP/loxP} and SNRK^{A-Cre} mice (Figure 2G–J). These results indicate that SNRK is required for maintaining insulin signaling and glucose utilization in both BAT and WAT.

3.2. Identification of PPP2R5D as a direct substrate of SNRK

Our previous studies demonstrated that SNRK is a key regulator of inflammation in WAT [8,9]. It is likely that impaired insulin signaling by SNRK ablation in WAT can be attributed to inflammation. However, in BAT of both SNRK^{-/-,A-Cre} and SNRK^{-/-,U-Cre} mice, no inflammation was observed yet insulin resistance exists, suggesting potential different mechanism. Therefore, two phosphoproteomic studies were conducted to understand the SNRK signaling pathway in BAT. In the first phosphoproteomic experiment, protein lysates were extracted from BAT of global SNRK deficient and littermate control pups at birth and differential protein phosphorylation was investigated. As shown in Figure 3A–B, the phosphorylation levels of three sites from two proteins (S570 and S584 on SNRK, and S80 on PPP2R5D, FDR adjusted P value < 0.05) were significantly and dramatically reduced by 99% in SNRK deficient BAT compared to BAT of littermate wild type pups. It is very surprising that only one protein was identified in addition to SNRK itself and SNRK was previously reported to be capable of auto-phosphorylation [18]. To confirm the result, primary brown adipocytes isolated from BAT of SNRK deficient pups were infected with adenovirus expressing green fluorescent protein or mouse SNRK. Overexpression of SNRK tremendously increased the phosphorylation on SNRK S570 and PPP2R5D S80 by more than 140 folds (Figure 3C, D). In addition to these two sites, phosphorylation level of PPP2R5D S81 was also increased by more than 140 folds (Figure 3D). PPP2R5D S80 was considered as the potential substrate of SNRK in BAT because this is the only overlapping site coming out of both phosphoproteomic studies. To understand whether PPP2R5D S80 could be a direct substrate of SNRK, an *in vitro* phosphorylation reaction was performed using purified recombinant human PPP2R5D full-length protein in the presence or absence of active purified recombinant human SNRK protein. The peptide fragment of human PPP2R5D containing S88–90 (human

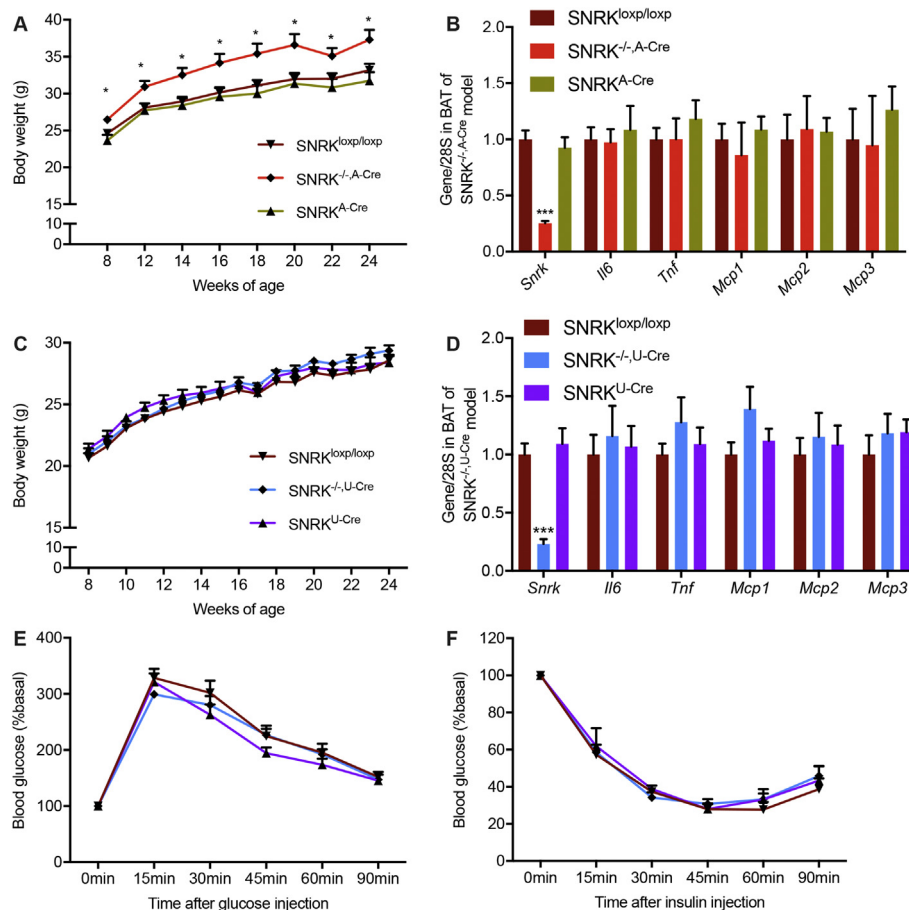


Figure 1: Characterization of adipose tissue-specific SNRK knockout (SNRK^{-/-}-A-Cre) and brown adipose tissue-specific SNRK knockout (SNRK^{-/-}-U-Cre) mice. A: Growth curve of SNRK^{-/-}-A-Cre and littermate control (SNRK^{loxp/loxp}, SNRK^{A-Cre}) mice (n = 6–12 per group). B: Expression of cytokine and chemokine genes in brown adipose tissue of SNRK^{-/-}-A-Cre and littermate control mice (n = 6–8 per group). C: Growth curve of SNRK^{-/-}-U-Cre and littermate control (SNRK^{loxp/loxp}, SNRK^{U-Cre}) mice (n = 8–13 per group). D: Expression of cytokine and chemokine genes in brown adipose tissue of SNRK^{-/-}-U-Cre and littermate control mice (n = 6 per group). E: Glucose tolerance test of SNRK^{-/-}-U-Cre and littermate control male mice (13-week old, n = 7 per group) fed a chow diet. F: Insulin tolerance test of SNRK^{-/-}-U-Cre and littermate control male mice (13-week old, n = 7 per group) fed a chow diet. Two-way repeated-measures ANOVA (SNRK KO × time) followed by Bonferroni *post hoc* test was used in Figure 1A,C, E & F, and one-way ANOVA followed by Bonferroni *post hoc* test in Figure 1B,D. **P* < 0.05, ****P* < 0.001 SNRK^{-/-}-A-Cre or SNRK^{-/-}-U-Cre vs their littermate controls. A-Cre, adiponectin-Cre; U-Cre, UCP-1-Cre; BAT, brown adipose tissue; IL, interleukin; TNF, Tumor Necrosis Factor; MCP, monocyte chemoattractant protein. Note: Unlike SNRK^{-/-}-U-Cre, SNRK^{-/-}-A-Cre mice exhibit glucose intolerant and insulin resistant compared with the littermate controls, which are reported previously [9].

PPP2R5D S88 is the equivalent of mouse PPP2R5D S80) could only be phosphorylated in the presence of SNRK, indicating that PPP2R5D is likely a direct substrate of SNRK.

PPP2R5D (also known as B'δ, B56δ, or PR61D) is one of the regulatory subunits of PP2A, which is a heterotrimeric protein serine/threonine phosphatase [19]. The fact that SNRK regulates phosphorylation of PPP2R5D leads to the hypothesis that SNRK may have an impact on PP2A activity. PP2A activities were then evaluated in both SNRK deficient adipocytes and adipose tissue. PP2A activities were found to significantly increase in both primary brown adipocytes isolated from SNRK deficient pups (Figure 4A) and cultured 3T3-L1 adipocytes with SNRK ablated by CRISPR technology (Figure 4B) compared to corresponding control cells. Enhanced PP2A activities were also observed in BAT of SNRK^{-/-}-U-Cre mice (Figure 4C) and in both WAT and BAT of SNRK^{-/-}-A-Cre mice (Figure 4D, E) compared to littermate control SNRK^{loxp/loxp} and SNRK^{U-Cre} or SNRK^{A-Cre} mice, respectively.

3.3. Effect of SNRK deficiency on the assembly of PP2A-PPP2R5D-AKT complex

PP2A functions as a heterotrimer, which consists of a structural A subunit, a catalytic C subunit, and regulatory B subunits. PP2A substrate specificity is determined by the incorporated regulatory subunit of the PP2A holoenzyme [20,21]. It has been shown that PP2A is involved in the regulation of AKT phosphorylation [22,23]. In the current study, SNRK deficiency was found to cause decreased AKT phosphorylation on S473 and T308 as well as decreased PPP2R5D phosphorylation on S80, which was accompanied by increased PP2A activity. It is reasonable to hypothesize that SNRK enhances insulin signaling by phosphorylating PPP2R5D on S80, which inhibits PP2A activation and therefore increases AKT phosphorylation on S473 and T308. To test whether SNRK deficiency affects coordinated assembly of the PP2A-PPP2R5D holoenzyme complex on AKT, endogenous AKT was immunoprecipitated from protein lysates of primary brown adipocytes. Both PPP2R5D and PP2AC were found to exist in the immuno-

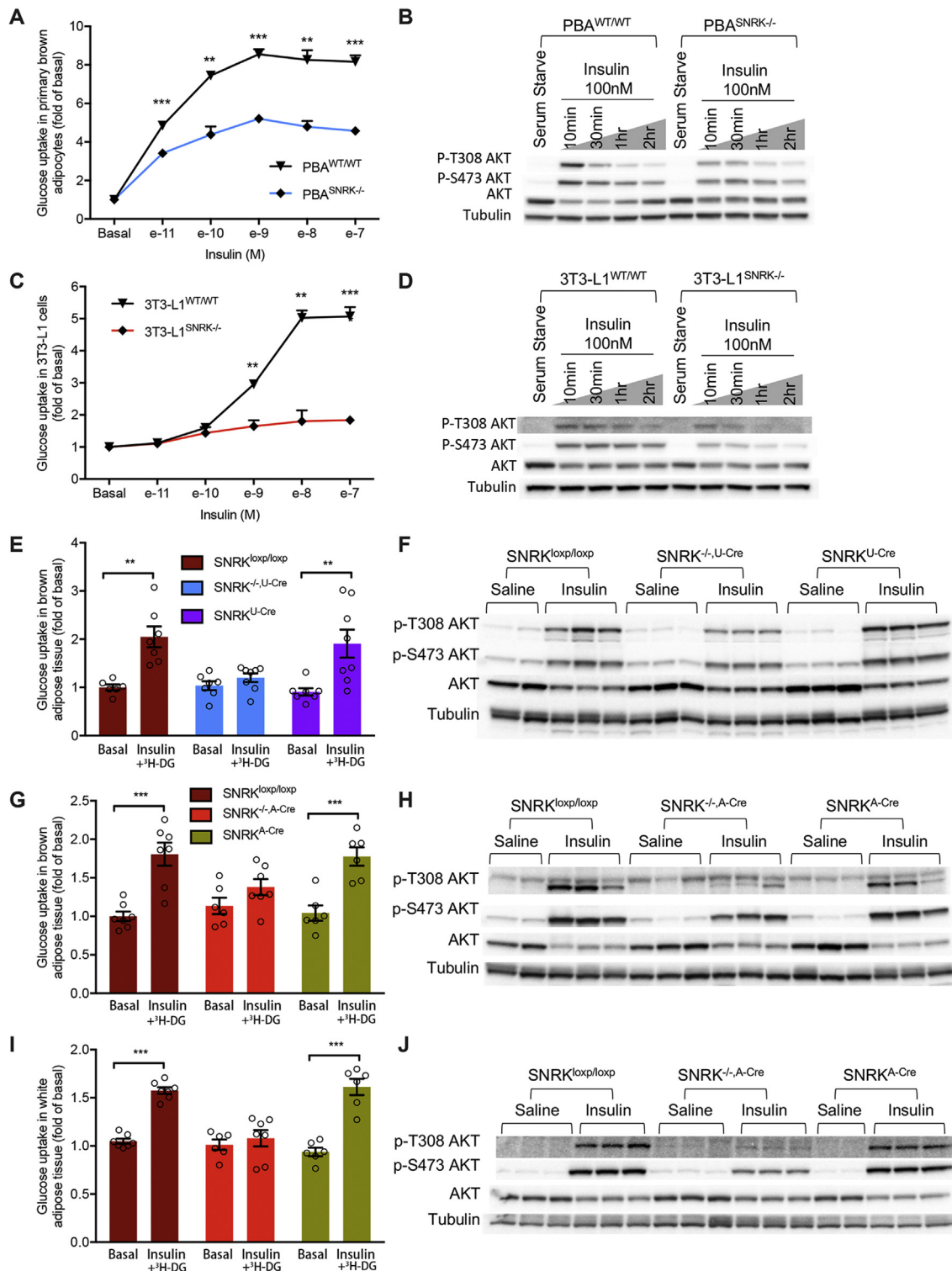


Figure 2: Attenuation of insulin-induced glucose uptake and AKT phosphorylation by SNRK deficiency in brown and white adipocytes as well as brown and white adipose tissues. A–B: Glucose uptake (A) and AKT phosphorylation (B) in insulin treated SNRK deficient primary brown adipocytes (PBA^{SNRK-/-}) and control wild type brown adipocytes (PBA^{WT/WT}) (n = 3 for A) derived from the same litter of pups. C–D: Glucose uptake (C) and AKT phosphorylation (D) in insulin treated SNRK CRISPR knockout 3T3-L1 cells (3T3-L1^{SNRK-/-}) and control cells (3T3-L1^{WT/WT}) (n = 3 for C). E–F: Glucose uptake (E) and AKT phosphorylation (F) in brown adipose tissue of SNRK^{-/-,U-Cre} and littermate controls (n = 6–8 for E) collected 10 min after insulin injection through portal vein. G–H: Glucose uptake (G) and AKT phosphorylation (H) in brown adipose tissue of SNRK^{-/-,A-Cre} and littermate controls (n = 6–7 for G) harvested 10 min after injection with insulin and (0.5 U/kg) and ³H-Deoxy-D-glucose (³H-DG, 130 uCi/kg) through portal vein. I–J: Glucose uptake (I) and AKT phosphorylation (J) in white adipose tissue of SNRK^{-/-,A-Cre} and littermate controls (n = 6–7 for I) harvested 10 min after injection with insulin and (0.5 U/kg) and ³H-DG (130 uCi/kg) through portal vein. Two-way repeated-measures ANOVA (SNRK KO × time) followed by Bonferroni *post hoc* test was used in Figure 2A,C, and two-way ANOVA (SNRK KO × insulin treatment) followed by Bonferroni *post hoc* test in Figure 2E,G&I. *P < 0.05, **P < 0.01, and ***P < 0.001 as indicated.

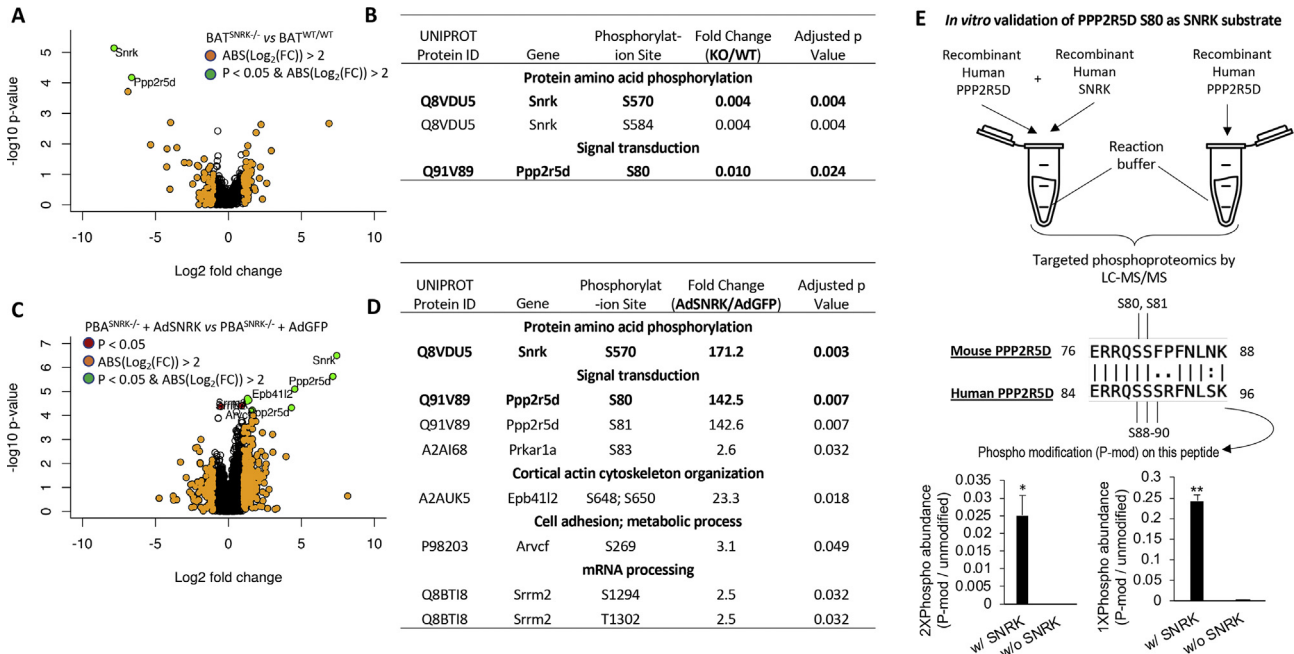


Figure 3: Identification of PPP2R5D as a potential novel substrate of SNRK in brown adipose tissue. A: Volcano plot representing the magnitude and significance of differential phosphopeptide abundance in brown adipose tissue of global SNRK knockout (BAT^{SNRK^{-/-}}) vs littermate wild type mice (BAT^{WT/WT}). B: List of phosphoproteins identified in Figures A with adjusted P value less than 0.05 and absolute value of Log₂(fold change) greater than 2. C: Volcano plot representing the magnitude and significance of differential phosphopeptide abundance in SNRK knockout primary brown adipocytes overexpressing SNRK (PBA^{SNRK^{-/-}} + AdSNRK) vs GFP (PBA^{SNRK^{-/-}} + AdGFP) through adenovirus-mediated infection (C) (n = 5). D: List of phosphoproteins identified in Figures C with adjusted P value less than 0.05 and absolute value of Log₂(fold change) greater than 2. The overlapped phosphorylation site, PPP2R5D S80, in panels B and D, was identified as the potential substrate of SNRK in brown adipose tissue. E: In vitro validation of PPP2R5D S80 as a direct SNRK substrate. The *in vitro* phosphorylation reaction was conducted using purified recombinant human PPP2R5D protein in the absence/presence of active SNRK protein. The human PPP2R5D S88 is equivalent to mouse PPP2R5D S80. *P < 0.05 and **P < 0.01 as indicated. ABS, absolute value; FC, fold change; Ad, adenovirus.

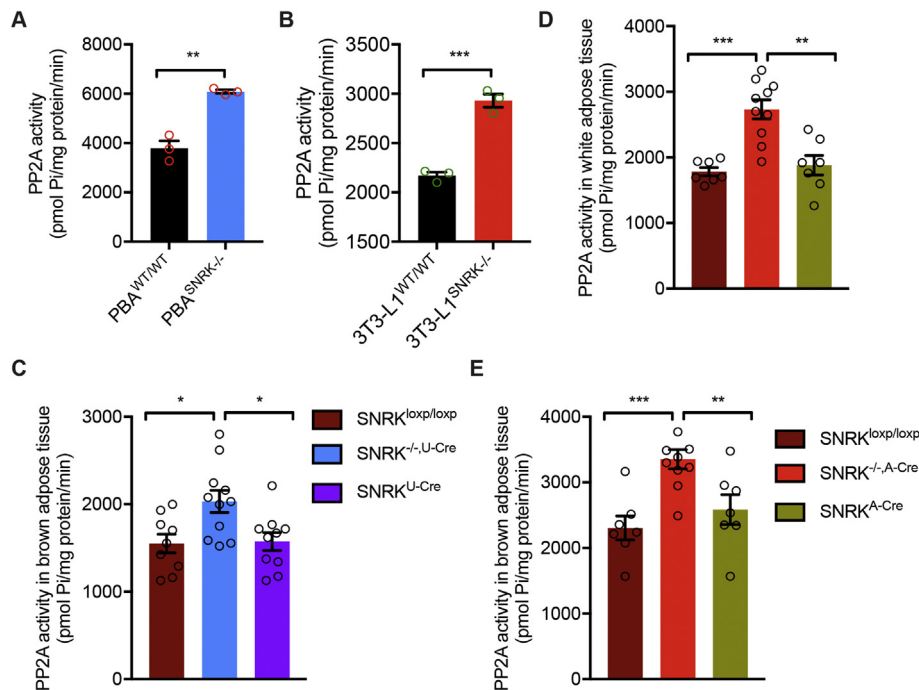


Figure 4: Enhanced PP2A activity in SNRK deficient brown and white adipose tissues. A: PP2A activity in SNRK knockout primary brown adipocytes (PBA^{SNRK^{-/-}}) and control cells (PBA^{WT/WT}). B: PP2A activity in SNRK knockout 3T3-L1 cells (3T3-L1^{SNRK^{-/-}}) and control cells (3T3-L1^{WT/WT}) (n = 3). C: PP2A activity in brown adipose tissue of SNRK^{-/-}, U-Cre and littermate controls (SNRK^{Loxp/Loxp} and SNRK^{U-Cre}) (n = 9–11). D–E: PP2A activity in white (D) and brown (E) adipose tissues of SNRK^{-/-}, A-Cre and littermate controls (SNRK^{Loxp/Loxp} and SNRK^{A-Cre}) (n = 7–10). *t* test was used in panels C & E, and one-way ANOVA followed by Bonferroni *post hoc* test in panels A, B, & D. *P < 0.05, **P < 0.01, and ***P < 0.001 as indicated.

complex pulled down by AKT antibody (Figure 5A). SNRK deletion constitutively increased the expression of PPP2R5D and the interaction of PP2A-PPP2R5D holoenzyme with AKT regardless of the insulin treatment in primary brown adipocytes (Figure 5A). The increased PP2A-AKT interaction in SNRK deficient adipocytes could be the mechanism preventing AKT from being phosphorylated and subsequently decreased insulin-induced glucose uptake. Increased interaction between SNRK and PP2A holoenzyme upon insulin treatment was observed in both WAT and BAT of control mice (Figure 5B, C). The increased expression level of PPP2R5D protein was also observed in BAT of both SNRK^{-/-,U-Cre} and SNRK^{-/-,A-Cre} mice compared to littermate control mice (Figure 5D, E). The elevation of PPP2R5D protein in WAT of SNRK^{-/-,A-Cre} mice was also observed (Figure 5F). The amount of PP2A holoenzyme that co-immunoprecipitates with AKT increased in BAT of SNRK^{-/-,U-Cre} mice and in both WAT and BAT of SNRK^{-/-,A-Cre} mice compared to littermate control mice (Figure 5D–F), indicating that SNRK deficiency leads to increased PP2A holoenzyme recruitment to AKT and therefore impairs insulin signaling.

3.4. Knockdown of PPP2R5D improves insulin sensitivity in adipocytes without SNRK expression

To further confirm the role that PPP2R5D plays in SNRK deficiency induced insulin resistance, PPP2R5D was knocked down in both PBA^{SNRK^{-/-}} and 3T3 L1^{SNRK^{-/-}} adipocytes. The knockdown efficiency was confirmed by real-time PCR (Figure 6A,D). PPP2R5D knockdown in PBA^{SNRK^{-/-}} and 3T3 L1^{SNRK^{-/-}} cells significantly decreased PP2A activity (Figure 6B,E) and increased insulin-induced glucose uptake (Figure 6C,F).

4. DISCUSSION

SNRK is the predominant kinase controlling carbohydrate metabolism in plants, and its role in metabolism in mammals is just beginning to be revealed. We are the first to investigate the roles of SNRK playing in adipose metabolism. Our previous reports have shown that SNRK can suppress inflammation in white adipocyte and WAT and is essential for maintaining BAT thermogenesis [8,9]. In this study, we also found that SNRK is indispensable in insulin signaling in both WAT and BAT since SNRK deficiency significantly inhibited insulin-stimulated AKT phosphorylation and glucose uptake. Utilizing the state-of-the-art phosphoproteomic studies, we identified a novel SNRK substrate, PPP2R5D, through which SNRK regulates PP2A activity and insulin-induced AKT phosphorylation and glucose uptake in adipose tissue. Recent literature has revealed the existence of BAT in adult humans. BAT has become an attractive target for treating obesity considering that the major function of BAT is to dissipate energy as heat through the uncoupling protein 1 (UCP1), and this function is usually compromised in obesity [4,24]. In addition to the role in control of thermogenesis, increasing evidence indicates that BAT also functions as a regulator of glucose homeostasis [25]. Glucose is taken up by BAT through two mechanisms, the insulin-dependent and the insulin-independent process. It has been shown that brown adipocytes and skeletal muscle cells share a common developmental origin [26]. The sensitivity of BAT to insulin is as high as that of skeletal muscle [27]. Specifically, glucose uptake in BAT can occur in response to sympathetic stimulation, such as cold exposure and β 3 adrenergic receptor activation, which is an insulin-independent process [27–30]. We

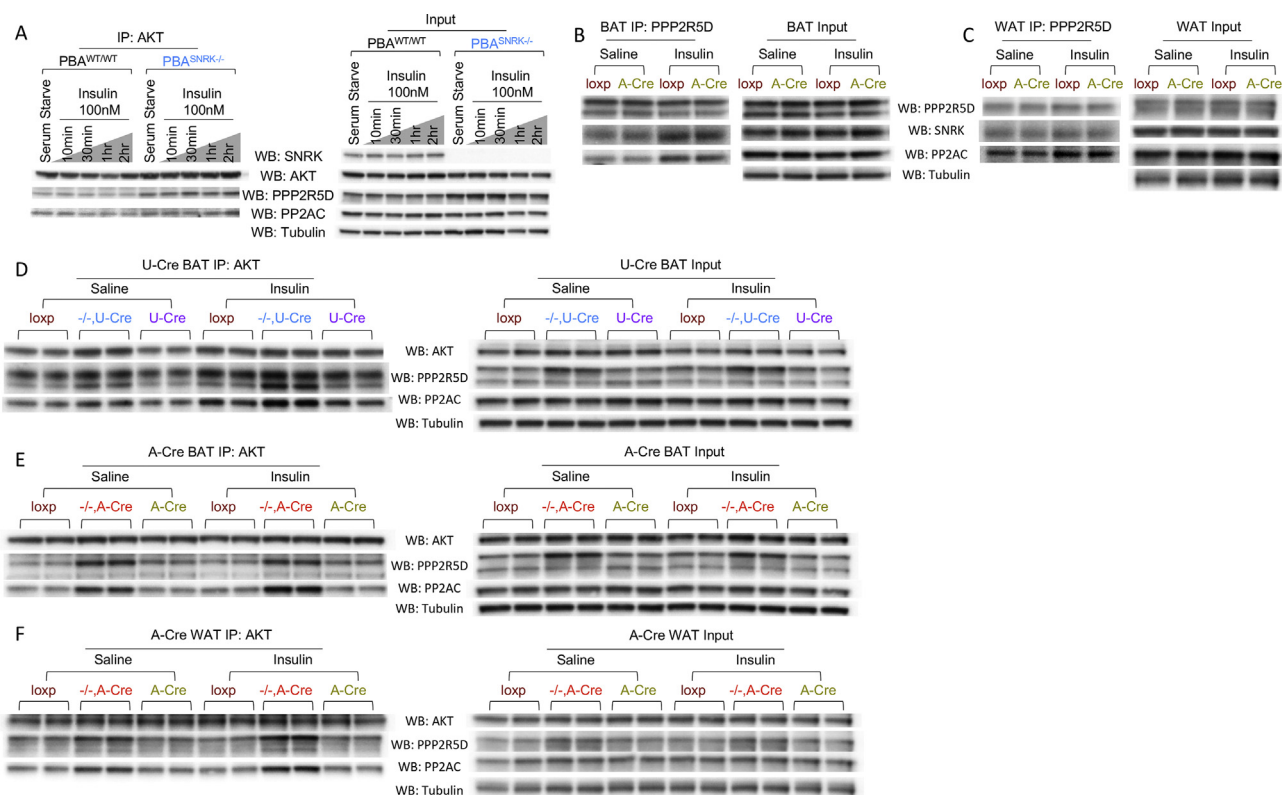


Figure 5: Increased AKT-PPP2R5D-PP2AC interaction in SNRK deficient brown and white adipose tissues. A: Co-immunoprecipitation of AKT, PPP2R5D, and PP2AC in SNRK knockout primary brown adipocytes (PBA^{SNRK^{-/-}}) and control cells (PBA^{WT/WT}). B–C: Co-immunoprecipitation of PPP2R5D, SNRK, and PP2AC in brown (B) and white (C) adipose tissues of SNRK^{loxp/loxp} and SNRK^{-/-,A-Cre} mice. D: Co-immunoprecipitation of AKT, PPP2R5D, and PP2AC in brown adipose tissue of SNRK^{-/-,U-Cre} and littermate controls. E–F: Co-immunoprecipitation of AKT, PPP2R5D, and PP2AC in brown (E) and white (F) adipose tissues of SNRK^{-/-,A-Cre} and littermate controls.

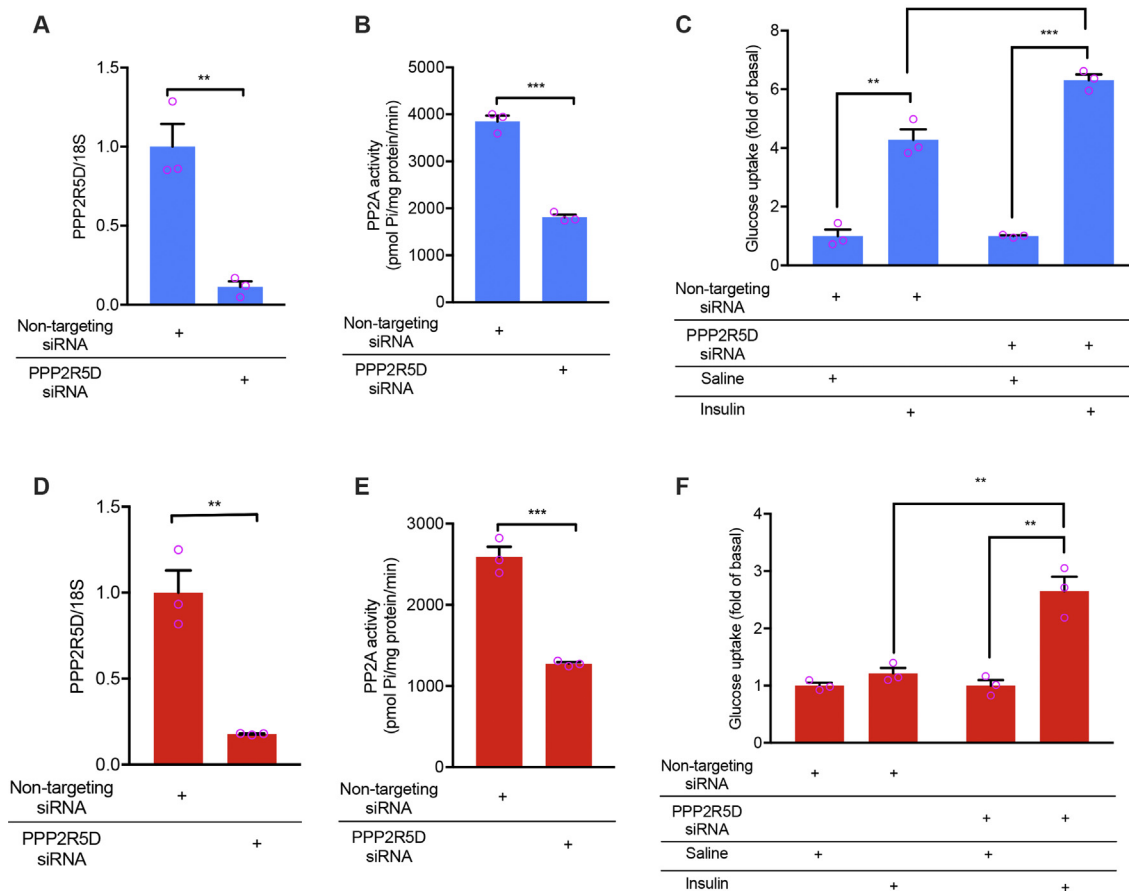


Figure 6: *Ppp2r5d* knockdown decreases PP2A activity and increases glucose uptake in SNRK knockout primary brown adipocytes and 3T3-L1 cells. A–C: PPP2R5D gene expression (A), PP2A activity (B), and glucose uptake (C) in SNRK knockout primary brown adipocytes (PBA^{SNRK-/-}) treated with *Ppp2r5d* siRNA or non-targeting siRNA (n = 3). D–F: *Ppp2r5d* gene expression (D), PP2A activity (E), and glucose uptake (F) in SNRK knockout 3T3-L1 cells (3T3-L1^{SNRK-/-}) treated with *Ppp2r5d* siRNA or non-targeting siRNA (n = 3). *t* test was used in panels A, B, D, & E, and two-way ANOVA followed by Bonferroni *post hoc* test in Figure 6C,F. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 as indicated.

aimed to dissect the role of SNRK in insulin resistance in WAT vs BAT; therefore, insulin was used to stimulate glucose uptake in this study. BAT is considered as a highly plastic metabolic organ that can regulate whole-body metabolism at a disproportionate scale even though its depot size is small. Some studies have shown that BAT activation by cold exposure [31] and β 3 adrenergic receptor agonist binding [32] as well as BAT transplantation [5,33,34] can increase peripheral insulin sensitivity and improve glucose tolerance. In our previous study using global SNRK knockout pups, we found that SNRK deficiency in BAT caused lower mitochondrial density and occasional ruptures of mitochondrial cristae and reduced UCP1 and PRDM16 mRNA expression by ~60% in BAT. SNRK deficiency in both WAT and BAT rendered them resistant to CL316,243-induced UCP1 expression and body weight reduction [9]. In the current study, we found that SNRK deficiency exclusively in BAT can inhibit insulin-induced AKT phosphorylation and glucose uptake in BAT; however, this is insufficient to affect whole-body glucose homeostasis, because the mouse model with SNRK deficiency in BAT only shows normal insulin sensitivity based on ITT and GTT assay. Notwithstanding, our findings cannot be interpreted as challenges to the importance of BAT in maintaining glucose homeostasis. The ability of BAT to regulate glucose metabolism is dramatically increased in its activation status [27], and the fact that impaired insulin signaling in BAT cannot affect systemic insulin sensitivity does

not mean BAT activation cannot improve whole body glucose homeostasis.

In our study, we found that SNRK deficiency in BAT dramatically inhibits insulin-stimulated glucose uptake as well as phosphorylation on T308 and S473 of AKT. AKT has been recognized an essential regulator of insulin-stimulated glucose uptake in both muscle and adipose tissue. Full activation of AKT depends on the phosphorylation on its regulatory residues T308 and S473 [35,36]. Phosphorylation on T308 and S473 of AKT upon insulin stimulation is controlled by kinases, such as PDK1 [37], and phosphatases, such as PP2A [22], PHLPP [38], and PP1 [39]. Our study clearly showed that SNRK is indispensable for an integrated insulin signaling pathway, although how SNRK regulates AKT phosphorylation was unclear. Through both loss-of-function and gain-of-function phosphoproteomic studies, we identified PPP2R5D S80 as a novel SNRK substrate. The finding was further validated in two ways, *in vitro* phosphorylation reaction using recombinant human SNRK and PPP2R5D and endogenous SNRK/PPP2R5D co-immunoprecipitation (co-IP) assay. The *in vitro* phosphorylation study indicates that SNRK can phosphorylate PPP2R5D directly. The co-IP assay results also support the putative SNRK-PPP2R5D interaction. PPP2R5D is one of the regulatory subunits of PP2A holoenzyme which was reported to negatively regulate AKT activity [23,40]. Therefore, we hypothesized that SNRK may regulate assembly of the PP2A

holoenzyme complex on AKT through PPP2R5D phosphorylation and subsequently affect insulin signaling and glucose uptake.

As a key serine–threonine phosphatase, PP2A counteracts most of the kinase-driven signaling pathways in mammalian cells [41]. PP2A holoenzyme is formed by three subunits, structure A subunit, catalytic C subunit, and regulatory B subunit that determine the substrate specificity. To date, 20 regulatory B subunits have been identified, which are encoded by 15 genes and classified into 4 families according to the sequence similarity [42]. Among them, it has been reported that PPP2R2A (B55 α , B α) [23], PPP2R5B (B56 β , B' β) [22], and PPP2R5D (B56 δ , B' δ) [40] can target PP2A holoenzyme to AKT. However, PPP2R2A guides PP2A holoenzyme to dephosphorylate AKT at T308 rather than S473, while the PP2A holoenzyme comprised of PPP2R5B or PPP2R5D can dephosphorylate AKT at both T308 and S473. Consistently, our study found that SNRK ablation in BAT dephosphorylates PPP2R5D S80, which led to increased PP2A activity, elevated PP2AC-AKT interaction, dampened phosphorylation on AKT T308 and S473 upon insulin stimulation, and decreased glucose uptake. We are the first to identify SNRK as a novel upstream kinase of PPP2R5D, which can also be phosphorylated by other kinases such as protein kinase A (PKA) [43] and C (PKC) [44].

In this study, we also compared the effects of SNRK deficiency on insulin signaling in BAT vs WAT. SNRK ablation can lead to defective insulin signaling in both BAT and WAT, albeit the systemic insulin resistance was not observed when SNRK was specifically knocked out in BAT. Taken together with the results from phosphoproteomics studies conducted in white and brown adipocytes, different substrates were identified for SNRK in distinct types of adipocytes, HDAC1 and EEF1D in white adipocytes [9] and PPP2R5D in brown adipocytes. Despite the fact that PPP2R5D was not identified in the phosphoproteomic study using SNRK knock down 3T3-L1 adipocytes, the PP2A activity and co-IP assay results indicate that PPP2R5D is also involved in SNRK

deficiency-induced insulin resistance in WAT. It is possible that the residual amount of SNRK in 3T3-L1 adipocytes expressing interfering RNA targeting SNRK is sufficient for maintaining PPP2R5D phosphorylation because only about 70% of SNRK was eliminated by RNA interference. Considering that HDAC1 and EEF1D mediate the SNRK-related inflammation process, it seems reasonable that HDAC1 and EEF1D were identified only in WAT but not in BAT, because SNRK deficiency causes elevated mRNA expression of cytokine and chemokine genes in WAT but not in BAT. The results from the current study and our previous study are summarized as a cartoon in Figure 7. SNRK regulates insulin signaling through PPP2R5D in BAT and HDAC1, EEF1D, and PPP2R5D in WAT.

5. CONCLUSION

In summary, we identify not only SNRK as a crucial regulator of insulin signaling in both BAT and WAT but also a novel SNRK-PPP2R5D-PP2AC-AKT pathway controlling insulin-regulated AKT phosphorylation and glucose uptake in adipose tissue. SNRK may represent a potential target for treating insulin resistance-related metabolic disorders.

AUTHOR CONTRIBUTION

JL and HX conceived the idea. JL took the lead to establish animal and cell models and conduct experiments. RA, SLai, and LL assisted in animal studies, phosphoproteomic studies, and co-immunoprecipitation assay. JL, HX, and SLiu wrote the manuscript. HX is the guarantor of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2019.07.009>.

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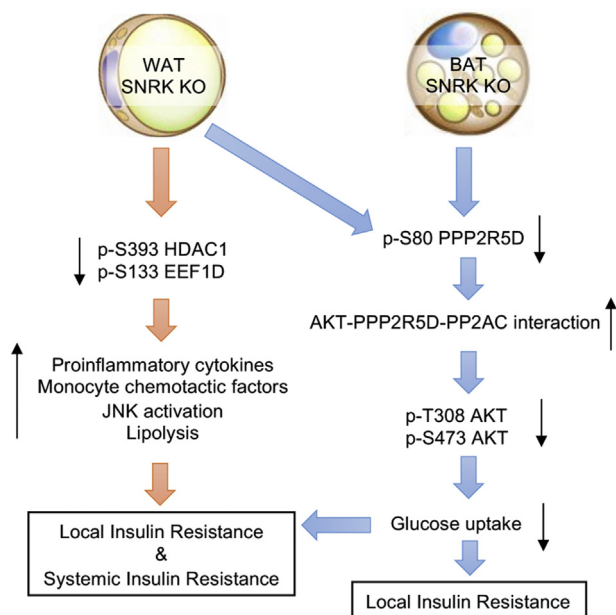


Figure 7: Hypothetical model of SNRK and insulin resistance. SNRK deficiency in white adipose tissue (WAT) causes both local and systemic insulin resistance through increasing inflammation [9] and decreasing insulin-induced glucose uptake. However, SNRK deficiency in brown adipose tissue (BAT) results in decreased insulin-induced glucose uptake only and local but not systemic insulin resistance. Yellow and blue arrows indicate inflammation and insulin signaling pathways, respectively.

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