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# Inhibition of insulin/PI3K/AKT signaling decreases adipose Sortilin 1 in mice and 3 T3-L1 adipocytes

Jibiao Li, Cheng Chen, Yuan Li, David J. Matye, Yifeng Wang, Wen-Xing Ding, Tiangang Li*

*Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66160, United States*

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ABSTRACT

Sortilin 1(Sort1) is a vesicle trafficking receptor that mediates protein sorting in the endocytic and exocytic pathways. Sort1 is a component of the GLUT4 storage vesicles in adipocytes and is also involved in the regulation of adipogenesis. Sort1 protein is reduced in adipose of obese mice and humans, but the underlying cause is not fully understood. Here we report that insulin/PI3K/AKT signaling cascade critically regulates adipose Sort1 protein abundance. Administration of a PI3K inhibitor rapidly decreased Sort1 protein but not mRNA in adipose of chow-fed mice. In 3 T3-L1 adipocytes, serum-starvation or inhibition of the PI3K/AKT signaling also decreased Sort1 protein without affecting Sort1 mRNA expression. Sort1 protein downregulation upon PI3K inhibition was blocked by pretreatment of MG132 but not Bafilomycin A1, suggesting that PI3K inhibition caused Sort1 degradation via the proteasome pathway. Using a phospho-specific Sort1 antibody, we showed that endogenous Sort1 was phosphorylated at S825 adjacent to the DXXLL sorting motif on the cytoplasmic tail. We demonstrated that mutagenesis that abolished Sort1 S825 phosphorylation decreased insulin-stimulated Sort1 localization on the plasma membrane and Sort1 protein stability in 3 T3-L1 adipocytes. However, endogenous Sort1 phosphorylation at S825 was not affected by insulin stimulation or by inhibition of PI3K. In conclusion, this study revealed an important role of insulin signaling in regulating adipose Sort1 protein stability, and further suggests that impaired insulin signaling may underlie reduced adipose Sort1 in obesity. The cellular events downstream of insulin/PI3K/AKT signaling that mediates insulin regulation of Sort1 stability requires further investigation.

## 1. Introduction

Sortilin 1 (Sort1) belongs to the family of vacuolar protein sorting 10 protein (VPS10P)-domain receptors [1–3]. Sort1 is a single-pass transmembrane protein that mediates intracellular vesicle trafficking in the endocytic or exocytic pathways [3]. In trafficking vesicles, the N-terminal luminal domain of Sort1 interacts with its protein ligands while the cytoplasmic domain of Sort1 facilitates the recruitment of adaptor proteins that are involved in vesicular trafficking. Sortilin 1 is expressed in many tissues including metabolically active tissues such as liver, muscle and adipose, immune cells such as macrophages and lymphocytes, and the central nerve system [2,4]. Sort1 has been shown to mediate the intracellular trafficking of proteins that are involved in distinct cellular pathways in various tissues and cell types [4–8]. Recent studies revealed a role of Sort1 in the regulation of lipoprotein metabolism and both genetic variations and pathological changes of Sort1 function may be linked to cardiovascular disease risk [9–14].

Obesity and diabetes are associated with impaired glucose uptake and metabolism in adipose tissue, which may be partially caused by insulin resistance and reduced GLUT4 expression [15]. In 3 T3-L1 adipocytes, Sort1 has been identified as a component of the GLUT4 storage vesicle (GSV) involved in insulin-dependent glucose uptake [4,5]. It has been shown that both the formation of GSVs and their insulin responsiveness require the presence of Sort1 in 3 T3-L1 adipocytes [16–18]. Insulin-stimulated glucose uptake was significant reduced in Sort1-deficient 3 T3-L1 adipocytes [16]. Mice lacking Sort1 maintained overall glucose homeostasis but showed reduced basal glycolytic activity in adipose tissue [19]. More recently, it was reported that increased Sort1 expression repressed adipogenesis by regulating the trafficking and function of delta-like 1 homologue (DLK1), an inhibitor of adipocyte differentiation [20,21]. Whole body Sort1 knockout mice were not obese [19,22]. Studies carried out in tissue

*Abbreviations:* Sort1, Sortilin1; VPS10P, vacuolar protein sorting 10 protein; GSV, GLUT4 storage vesicle; TNFα, tumor necrosis factor α; GGA2, Golgi–associated, γ-adaptin ear–containing, ARF-binding protein-2; CK2, casein kinase 2; TBCA, Tetrabromocinnamic acid

* Corresponding author: Department of Pharmacology, Toxicology and Therapeutics, The University of Kansas Medical Center, 3901 Rainbow Blvd, Kansas City, KS 66160, United States.

*E-mail address:* tli@kumc.edu (T. Li).

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specific Sort1 knockout mice are still needed to better define the role of Sort1 in regulating adipose biology and function. In addition, it has been reported that adipose Sort1 protein abundance was reduced in obese mice and obese humans, but the underlying mechanisms require further investigation [23]. This study identified that insulin signaling through the PI3K/AKT cascade played an important role in regulating Sort1 protein stability in adipose tissue of mice and 3 T3-L1 adipocytes, which suggests that impaired insulin signaling may be a possible cause of decreased adipose Sort1 in obesity.

## 2. Materials and methods

### 2.1. Reagents

Antibodies against Sort1 (ab16640; Lot: GR64653-1), GLUT4 (ab48547; Lot: Ab654) and α-Tubulin (ab7291; Lot: GR122217-1) were from Abcam (Cambridge, MA). Antibodies against phospho-AKT (Ser-473; 4060; Lot: 19), AKT (4691; Lot: 17), ubiquitin (3933), LC3B (3868, Lot: 9), and Histone 3 (9717; Lot: 8), TNFα and wortmannin were purchased from Cell Signaling Technology (Danvers, MA). Antibody against GGA2 (H-175, sc-30,103; Lot: K0305) was purchased from Santa Cruz Biotechnology (Dallas, Texas). Antibodies against FLAG (M2) (F1804; Lot: SLBJ4607V) and Actin (A5441; Lot: 063 M4808), Anti-FLAG (M2) magnetic beads, AKT1/2 inhibitor VIII, cycloheximide, chloroquine, Bafilomycin A1, MG132, and TBCA were purchased from Sigma (St. Louis, MO). The antibody against phospho-Sort1 (S825) was developed by Epitomics (Burlingame, CA). Insulin was purchased from Eli Lilly and Company (Indianapolis, IN). PX866 was purchased from Cayman Chemical (Ann Arbor, MI). Alexa Fluor 488 IgG (A-21202; Lot: 1,572,559) and Alexa Fluor 594 (A-21207; Lot: 1,558,726) were purchased from Life Technologies (Grand Island, NY).

### 2.2. Mice

Male 8 weeks old C57BL/6 J mice and *Ob/Ob* mice were purchased from the Jackson Lab (Bar Harbor, ME). Mice were housed under a normal light-dark cycle (light on from 6 am–7 pm) with free access to food and water. *Ob/Ob* mice were sacrificed at 12 weeks of age after overnight (5 pm − 9 am) fasting. C57BL/6 J mice at 10 weeks of age were fed a Western diet (TD.88137) purchased from Envigo (Indianapolis, IN), and sacrificed after overnight (5 pm–9 am) fasting. Px866 was dissolved in sterile 1 × PBS with 5% ethanol. At around 9 am, Px866 was administered to 10-week old non-fasted C57BL/6 J mice through intraperitoneal injection at 8 mg/kg in a 100-μl volume [24]. Control mice were injected with vehicle. Mice were then fasted for 8 h. A drop of blood was collected from the tail and used for measurement of blood glucose with a glucometer. Mice were then sacrificed. All animal protocols were approved by the Institutional Animal Care and Use Committee.

### 2.3. Cell culture

Mouse 3 T3-L1 preadipocytes were a gift from Dr. Gökhan Hotamisligil (Harvard University School of Public Health). Differentiation was induced by culturing preadipocytes in DMEM supplemented with 10% bovine calf serum, 0.5 mM isobutyl methyl xanthine, 10 μM dexamathasone, 5 μg/ml insulin and 1 μM thiazolidinedione for 3 days, after which cells were cultured in maintenance medium (DMEM with 10% FBS, 1% P/S and 5 μg/ml insulin). Lenti-WT-Sort1-FLAG and Lenti-S825A-Sort1-FLAG (C-terminal-tagged) were generated by Capital Biosciences Inc. (Gaithersburg, MD). Heterogeneous pools of stable 3 T3-L1 cells were generated by selecting infected cells with 2.5 μg/ml puromycin. Stable cells were maintained in 2 μg/ml puromycin-containing DMEM in further experiments.

### 2.4. Western blotting

Cell lysates or tissue homogenates were prepared in 1 × RIPA buffer containing 1% SDS and protease inhibitor cocktail Sigma (St. Louis, MO). Lysates were incubated on ice for 30 min followed by brief sonication. After centrifugation, supernatant was transferred to a new tube and protein concentrations were determined by a BCA assay kit (ThermoFisher Scientific, Waltham, MA). Lysates containing equal amount of protein were used for SDS-PAGE and Western blotting. Densitometry was performed with ImageJ software and normalized to loading controls unless noted otherwise.

### 2.5. Co-immunoprecipitation assay

Differentiated 3 T3-L1 cells with stable expression of WT or S825A Sort1-FLAG were lysed in modified RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitor. Sort1-FLAG was precipitated with Anti-FLAG (M2) magnetic beads. Differentiated 3 T3-L1 cells that did not express Sort1-FLAG were used in the immunoprecipitation as negative controls. Precipitated Sort1-FLAG, GGA2 and GLUT4 were detected by Western blotting.

### 2.6. Real-time PCR

Total RNA was isolated with Tri-reagent (ThermoFisher Scientific, Waltham, MA). Real-time PCR were performed with SYBR master mix (Bio-Rad Laboratories Inc., Hercules, CA). Amplification of 18S was used as an internal control. Relative mRNA expression was quantified using the comparative CT (Ct) method and expressed as $2^{-\Delta\Delta Ct}$.

### 2.7. Immunofluorescent confocal microscopy

Cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% tween-20 and 0.3 M glycine. Primary antibodies and Alexa Fluor-conjugated secondary antibodies were used for staining. Images were taken with a Leica DM550Q confocal microscope and acquired with LAS AF software (Leica Microsystems Inc., Buffalo Grove, IL).

### 2.8. Surface biotinylation assay

Biotinylation assay was performed with Pierce Cell Surface Protein Isolation Kit (Cat#: 89,881) purchased from ThermoFisher Scientific (Waltham, MA) per manufacturer's instruction. Briefly, cells were immediately washed with ice-cold 1 × PBS after treatments and incubated in Sulfo-NHS-SS-Biotin solution at 4 °C for 30 min. Biotinylated proteins were purified from cell lysates with NeutrAvidin Agarose-containing columns. Biotinylated protein and flow-through were used for Western blotting.

### 2.9. Statistical analysis

Results were expressed as mean ± SE or mean ± S.D. as noted in figure legend. Statistical analysis was performed by Student's *t*-test. A $p < 0.05$ was considered statistically significant.

## 3. Results and discussion

### 3.1. Blocking the PI3K/AKT pathway causes Sort1 downregulation in mouse adipose tissue

Consistent with previous reports [19,23], we found that adipose Sort1 protein was significantly decreased in genetic obese *Ob/Ob* mice and in Western diet-induced obese mice (Fig. 1A, C). The Sort1 mRNA tended to be lower in obese mice but the reduction was not statistically significant (Fig. 1B, D). Similarly, a previous study suggested that elevated tumor necrosis factor α (TNFα) played a role in mediating the

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