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

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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 3T3-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 3T3-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.

Keywords

insulin resistance; diabetes; obesity; glucose metabolism; protein trafficking

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Author contributions

J.L., C.C., Y.L. D.M. and Y.W. designed and performed the experiments and analyzed the data. W.X.D. and T.L. supervised the study, designed and analyzed the data. T.L. wrote the manuscript.

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 3T3-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 3T3-L1 adipocytes (16–18). Insulin-stimulated glucose uptake was significantly reduced in Sort1-deficient 3T3-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 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 3T3-L1 adipocytes, which suggests that impaired insulin signaling may be a possible cause of decreased adipose Sort1 in obesity.

2. Materials and Methods

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), TNF α and wortmannin were purchased from Cell Signaling Technology (Danvers, MA). Antibody against GGA2 (H-175, sc-30103; Lot: K0305) was purchased from Santa Cruz Biotechnology (Dallas, Texas). Antibodies against FLAG (M2) (F1804; Lot: SLBJ4607V) and Actin (A5441; Lot: 063M4808), 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: 1572559) and Alexa Fluor 594 (A-21207; Lot: 1558726) were purchased from Life Technologies (Grand Island, NY).

Mice

Male 8 weeks old C57BL/6J 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/6J 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/6J mice through intraperitoneal injection at 8 mg/kg in a 100- μ l 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.

Cell culture

Mouse 3T3-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 μ M dexamethasone, 5 μ g/ml insulin and 1 μ M thiazolidinedione for 3 days, after which cells were cultured in maintenance medium (DMEM with 10% FBS, 1% P/S and 5 μ g/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 3T3-L1 cells were generated by selecting infected cells with 2.5 μ g/ml puromycin. Stable cells were maintained in 2 μ g/ml puromycin-containing DMEM in further experiments.

Western blotting

Cell lysates or tissue homogenates were prepared in 1X 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.

Co-immunoprecipitation assay

Differentiated 3T3-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 3T3-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.

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^{-Ct} .

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).

Surface biotinylation assay

Biotinylation assay was performed with Pierce Cell Surface Protein Isolation Kit (Cat#: 89881) purchased from ThermoFisher Scientific (Waltham, MA) per manufacturer's instruction. Briefly, cells were immediately washed with ice-cold 1x 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.

Statistical analysis

Results were expressed as mean \pm SE or mean \pm 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, 1C). The Sort1 mRNA tended to be lower in obese mice but the reduction was not statistically significant (Fig 1B, 1D). Similarly, a previous study suggested that elevated tumor necrosis factor α (TNF α) played a role in mediating the transcriptional inhibition of adipose Sort1 in obese mice, but Sort1 protein appeared to be reduced to a much larger extent than Sort1 mRNA in obese mice (23). Feeding mice a Western diet for 1 week decreased adipose Sort1 protein but not mRNA (Fig 1E, 1F), suggesting that adipose Sort1 started to decrease at the early stage of diet-induced obesity development. A previous study demonstrated that challenging mice a high fat diet for 3 days was sufficient to cause early onset insulin resistance in adipose due to acute lipid overload (25). Currently, the cause of adipose Sort1 downregulation in obesity is not clear. To test if adipose Sort1 downregulation could potentially be due to decreased insulin/PI3K/AKT signaling in obese mice, we

injected chow-fed C57BL/6J mice with a PI3K inhibitor Px866 or vehicle and mice were sacrificed 8 h post injection. This treatment significantly decreased the phosphorylation levels of AKT in the white adipose of mice (Fig 2A), confirming effective inhibition of the PI3K/AKT signaling. Px866 significantly decreased Sort1 protein (Fig 2A) without altering Sort1 mRNA expression (Fig 2B). As positive controls, GLUT4 protein was decreased and plasma glucose was increased upon Px866 treatment (Fig 2A, 2C). These results suggest that chronically attenuated PI3K/AKT signaling as a result of insulin resistance may be a potential mechanism underlying lower adipose Sort1 protein abundance in obese mice.

3.2. Inhibition of PI3K or AKT decreases Sort1 protein abundance in 3T3-L1 adipocytes

We next elaborated on this finding in differentiated 3T3-L1 adipocytes *in vitro*. It has been reported that undifferentiated 3T3-L1 fibroblast cells do not express Sort1, while Sort1 mRNA and protein are highly expressed when these cells differentiated into adipocytes (4, 26). Consistently, we found that the expression of Sort1 reached relatively constant levels after 6 days post induction of differentiation (Fig 3A). Cells between 8–11 days post induction of differentiation were used for the following experiments. Consistent with our *in vivo* observations, we found that treating differentiated 3T3-L1 adipocytes with the PI3K inhibitor wortmannin rapidly decreased Sort1 protein without affecting Sort1 mRNA (Fig 3B, 3C). Wortmannin also decreased Sort1 protein in cells pretreated with cycloheximide which blocked protein synthesis (Fig 3D), suggesting a posttranslational mechanism of Sort1 downregulation. Increased cytokines are known to play an important role in inhibiting adipose insulin signaling in obesity (27). We also found that Sort1 protein was decreased in 3T3-L1 adipocytes only after 24 h or longer TNF α treatment, which correlated with decreased AKT phosphorylation (Fig 3E). Consistent with a previous report (23), TNF α also decreased Sort1 mRNA expression, but to a less extent than Sort1 protein (Fig 3F). Culturing differentiated adipocytes in serum-free medium overnight decreased Sort1 protein (Fig 4A), while addition of insulin into the culture medium increased Sort1 protein, but not Sort1 mRNA, in overnight serum-starved cells (Fig 4B, 4C). Insulin induction of Sort1 protein was blocked by either wortmannin or an AKT inhibitor (Fig 4D, 4E). Wortmannin-induced Sort1 downregulation was prevented by the proteasome inhibitor MG132, but not by the lysosome inhibitor Bafilomycin A1 (Fig 4F, 4G). Ubiquitinated proteins and LC3B were measured as controls in these experiments. These results suggest that inhibition of cellular PI3K signaling probably promoted proteasome-dependent Sort1 degradation.

3.3. S825 phosphorylation of Sort1 was not affected by insulin or PI3K/AKT inhibition

The mechanism underlying the rapid posttranslational downregulation of adipose Sort1 caused by blocking PI3K/AKT signaling is still not clear. Recently, we and others have identified a putative Sort1 phosphorylation site serine-825 (S825) via proteomics approaches (24, 28, 29). S825 is located on the cytoplasmic tail of Sort1 immediately adjacent to a conserved DXXLL motif (S⁸²⁵DEDLL in Sort1) that mediates Sort1 interaction with the trafficking adaptor Golgi-associated, γ -adaptin ear-containing, ARF-binding protein-2 (GGA2) (28, 30, 31). Abolishing S825 phosphorylation by S-A mutagenesis appeared to alter Sort1 cellular localization in vascular smooth muscle cells (29) and decrease protein stability in liver cells (24). In differentiated 3T3-L1 adipocytes stably expressing WT Sort1-FLAG or S825A Sort1-FLAG, we found that WT Sort1-FLAG showed minimal reduction in

protein abundance after protein synthesis was blocked by cycloheximide over a 12-h period, suggesting that Sort1 is a relatively stable protein in 3T3-L1 adipocytes (Fig 5A). In contrast, S825A Sort1-FLAG protein showed a faster degradation rate after cycloheximide treatment (Fig 5A). S825 is a consensus casein kinase target and was shown to be phosphorylated by casein kinase 2 (CK2) and the Golgi-localized casein kinase the Family with sequence similarity 20, member C (Fam20C) in *in vitro* kinase assays (28, 29). Although CK2 was generally thought to be constitutively active in various cell types (32), some studies also suggested that CK2 was rapidly activated by insulin, insulin-like growth factor-I and epidermal growth factor in 3T3-L1 adipocytes (33, 34). Activated CK2 may in turn promote cell proliferation in response to growth factor signaling (35, 36). These previous findings raise the question whether S825 phosphorylation is involved in insulin/PI3K/AKT regulation of Sort1 in adipocytes.

To better address these questions, we developed antibodies specifically detecting S825 phosphorylated Sort1. Fig 5B shows that in undifferentiated 3T3-L1 cells that did not express endogenous Sort1, phospho-Sort1 antibodies only detected WT Sort1-FLAG, but not S825A Sort1-FLAG. These results validated the specificity of the phospho-Sort1 antibodies. We next treated differentiated 3T3-L1 adipocytes with a CK2 inhibitor Tetrabromocinnamic acid (TBCA) and measured endogenous Sort1 phosphorylation at S825 (37). However, TBCA treatment for 2 h and 4 h did not affect S825 phosphorylation (Fig 5C). One possible explanation may be the existence of enzyme redundancy so that kinases other than CK2 may also phosphorylate S825 in 3T3-L1 adipocytes. We next investigated if S825 phosphorylation was affected by insulin/PI3K signaling. In undifferentiated 3T3-L1 cells that did not express endogenous Sort1, insulin treatment did not further increase the S825 phosphorylation of WT Sort1-FLAG (Fig 5D). In differentiated 3T3-L1 adipocytes, phosphorylated endogenous Sort1 was readily detectable in overnight serum-starved cells (Fig 5E). However, insulin increased the phospho-Sort1 and total Sort1 to similar extent, and the ratio of phospho-Sort1 to total Sort1 was not altered by insulin treatment over the course of 6 h (Fig 5E, 5F). Furthermore, treating differentiated 3T3-L1 adipocytes with wortmannin or AKT inhibitor for 2 h rapidly decreased both phosphorylated Sort1 and total Sort1 to similar extent (Fig 5G). In summary, our results confirmed that endogenous Sort1 was phosphorylated at S825 in 3T3-L1 adipocytes. The S825 phosphorylation was readily detectable in several experimental conditions, which left an impression that a relatively high percentage of endogenous Sort1 may be phosphorylated at S825 in 3T3-L1 adipocytes. It may be speculated that the constitutive activity of CK2 and other unidentified kinases phosphorylate Sort1 at S825 at high baseline levels to maintain Sort1 protein stability and constitutive trafficking function in cells (32, 35, 36).

3.4. Abolishing phosphorylation of S825 prevented insulin-stimulated Sort1 plasma membrane localization

It was suggested that phosphorylation of the serine residue immediately adjacent to the DXXLL di-leucine motif in many trafficking receptors regulates di-leucine motif interaction with the trafficking adaptor GGA2 (28, 30, 31). We next investigated if S825 phosphorylation is involved in insulin-regulated Sort1 plasma membrane localization. In differentiated 3T3-L1 adipocytes expressing WT Sort1-FLAG or S825A Sort1-FLAG, both

WT Sort1 and S825A Sort1 were primarily visualized as perinuclear puncta that substantially co-localized with GLUT4 (Fig 6A). Some Sort1 puncta also dispersed throughout the cells and showed co-localization with GLUT4 (Fig 6A). By using surface biotinylation assays, we were able to detect rapid plasma membrane enrichment of endogenous Sort1 in insulin-stimulated 3T3-L1 adipocytes (Fig 6C), which was consistent with a previous report (5). This experimental approach was used to further investigate the plasma translocation of WT and S825A Sort1-FLAG in response to insulin. Similar to endogenous Sort1, plasma membrane localization of WT Sort1-FLAG was stimulated by insulin (Fig 6D). In contrast, S825A Sort1-FLAG membrane localization was not enriched after insulin stimulation (Fig 6E). Longer insulin stimulation for 15 min decreased S825A Sort1-FLAG amount on the cell surface (Fig 6E). It is not known if this could be a combined result of S825A Sort1-FLAG endocytosis without stimulated membrane enrichment. Consistent with the lack of insulin-stimulated membrane enrichment of S825A Sort1-FLAG, the amount of GGA2 that co-precipitated with S825A Sort1-FLAG was also less than that co-precipitated with WT-Sort1-FLAG in a co-immunoprecipitation assay (Fig 6F). These results showed that abolishing S825 phosphorylation reduced Sort1 interaction with trafficking adaptor GGA2 and insulin-dependent Sort1 trafficking to the plasma membrane.

Despite being a relatively stable protein (Fig 5A), we have provided several lines of *in vitro* and *in vivo* evidence to show that Sort1 downregulation in response to inhibition of PI3K/AKT signaling was strong and rapid, which suggests stimulated Sort1 protein degradation under this condition. The downstream mechanism that caused such rapid Sort1 destabilization remains to be determined. The parallel changes of phosphorylated and total Sort1 suggest that alteration of S825 phosphorylation did not appear to be an event prior to total Sort1 changes caused by insulin treatment or by PI3K/AKT inhibition. Therefore, although protein phosphorylation is considered as a common mechanism by which cellular signaling regulates protein stability and degradation, our results do not support a potential mechanism by which insulin/PI3K/AKT signaling regulates Sort1 protein stability via altering Sort1 S825 phosphorylation. However, by studying the functional link of S825 phosphorylation to Sort1 stability and trafficking, we learned that abolishing S825 phosphorylation decreased Sort1 trafficking to the plasma membrane upon insulin stimulation, which correlated with reduced Sort1 protein stability. This observation may indirectly imply a possibility that cellular events that alter Sort1 trafficking process may be linked to Sort1 degradation. This is also supported by a recent study showing that altered AP-1-dependent Sort1 trafficking also affected cellular Sort1 protein degradation and Sort1 protein abundance without changes in Sort1 mRNA expression in adipocytes and in adipose tissue of mice (20). In addition, it is known that functional GSV formation depends on the key protein components to form a stable complex, and some experimental conditions that reduced GSV formation, such as by reducing Sort1 expression, also destabilized GLUT4 and caused GLUT4 degradation in adipocytes (16, 38). Whether Sort1 protein is also stabilized after it is incorporated into protein complex requires further investigation. As many protein components in GSV are insulin-regulated proteins (38), further studies are needed to investigate if inhibition of insulin/PI3K/AKT signaling somehow impairs processes such as Sort1-containing protein complex formation and/or vesicular trafficking process, which leads to Sort1 destabilization.

4. Conclusions

The major finding of this study is that the insulin/PI3K/AKT signaling cascade plays an important role in regulating adipose Sort1 at posttranslational levels, which suggests that impaired insulin signaling may be an underlying mechanism that results in lower adipose Sort1 protein in obese mice and obese humans (23). Adipose Sort1 downregulation occurred at early stage of diet-induced obesity, which was consistent with previous findings that challenging mice a high fat diet for 3 days was sufficient to cause early onset insulin resistance in adipose due to acute lipid overload (25). Sort1 is a component of GSV in adipocytes, and Sort1 loss-of-function was shown to decrease insulin-dependent glucose uptake into the adipocytes (16). Therefore, down-regulation of adipose Sort1 may be implicated in altered adipose glucose metabolism under obesity and insulin resistant conditions. However, the *in vivo* role of Sort1 in adipose glucose metabolism seems to be more complex as mice lacking Sort1 globally showed reduced basal adipose glycolytic activity but normal insulin-stimulated adipose glucose uptake (19). Sort1 has also been shown to inhibit adipogenesis by regulating the intracellular trafficking of DLK1, a receptor that inhibits adipogenesis (20, 21), and increased adipose Sort1 expression decreases adipogenesis and adipose mass. This finding raises another question whether downregulation of adipose Sort1 in obesity helps promote adipose differentiation in response to higher dietary lipid uptake. We recently reported that Sort1 knockout mice gained similar body weight as their WT counterparts when placed on a high fat diet (19). However, study of the genotype-dependent effects in high fat diet feeding model is limited due to the rapid and strong downregulation of Sort1 in the adipose tissue of WT control mice. Whether adipose-specific Sort1 transgenic mice have reduced adipogenesis and lower fat mass remains to be determined. Furthermore, it should be noted that Sort1 has also been implicated in various other cellular pathways in adipocytes such as mediating the endocytic targeting of lipoprotein lipase (7) and adiponectin (39) and neurotensin signaling (40). However, the functional roles of Sort1 in regulating these pathways are less well characterized in adipocytes and *in vivo*. Because findings from this study suggest that adipose Sort1 is highly sensitive to downregulation under insulin resistant conditions, the potential pathophysiological significance of decreased Sort1 in other aspects of adipose function in obesity and diabetes needs to be further defined by future studies.

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Abbreviations

Sort1	Sortilin1
VPS10P	vacuolar protein sorting 10 protein
GSV	GLUT4 storage vesicle

DLK1	delta-like 1 homologue
TNFα	tumor necrosis factor α
GGA2	Golgi-associated, γ -adaptin ear-containing, ARF-binding protein-2
CK2	casein kinase
TBCA	Tetrabromocinnamic acid

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Highlights

- Adipose Sortilin 1 protein is strongly downregulation in obesity.
- Insulin/PI3K/AKT signaling critically controls Sort1 protein abundance in adipocytes
- Sortilin 1 S825 phosphorylation regulates Sort1 trafficking function in adipocytes

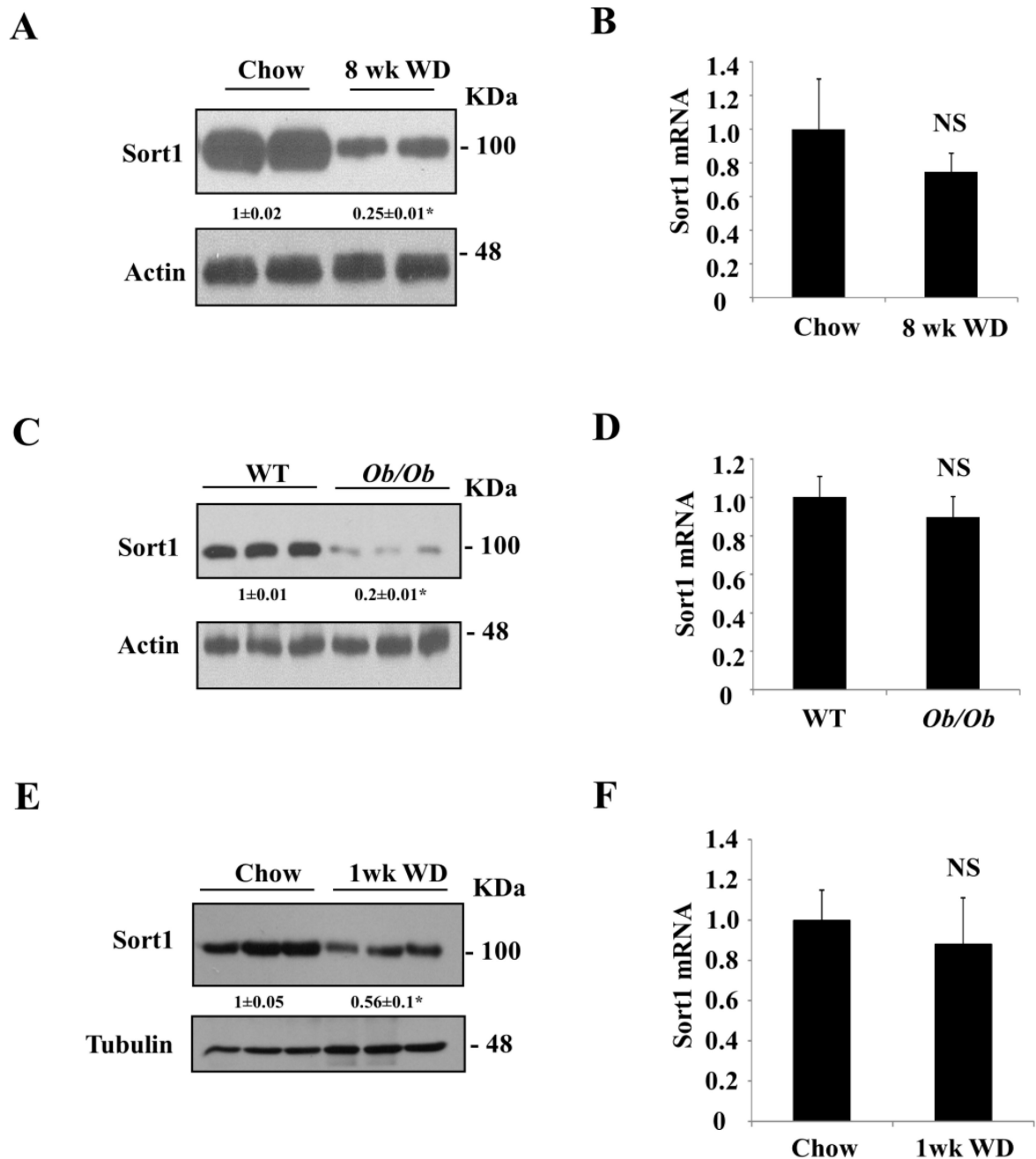


FIGURE 1. Sort1 protein was decreased in white adipose of obese mice

A, B. Sort1 protein and mRNA levels in white adipose of C57BL/6J mice fed a Western diet for 8 weeks. A representative bot was shown. Densitometry values are expressed as mean \pm SE (n=5–6). **C, D.** Sort1 protein and mRNA levels in white adipose of WT C57BL/6J and *Ob/Ob* mice. Densitometry values are expressed as mean \pm SE (n=3). **E, F.** Sort1 protein and mRNA levels in white adipose of C57BL/6J mice fed a Western diet for 1 wk. Densitometry values are expressed as mean \pm SEM (n=3). For all mRNA measurement, results are expressed as mean \pm SE (n=4–5). “*”, p< 0.05 vs. chow-fed controls. NS, not significant.

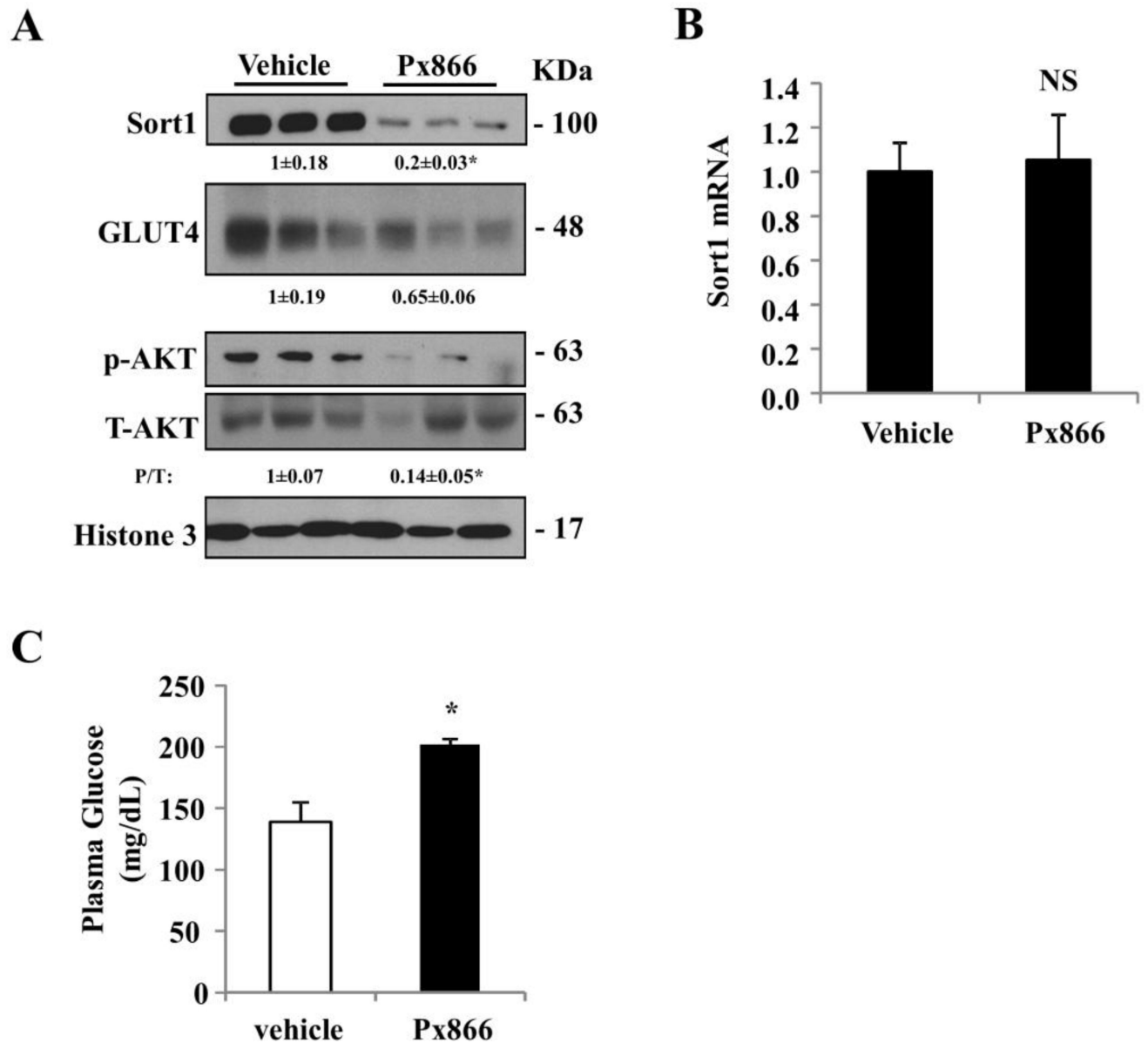


FIGURE 2. Blocking PI3K/AKT signaling caused downregulation of Sort 1 in mice

A. Sort1 protein in white adipose of mice 8 h post injection of Pxx866 or vehicle. Relative Sort1 band intensity values (mean ± SE) are shown (n=3). **B.** Sort1 mRNA in white adipose of mice 8 h post injection of Pxx866 or vehicle. n=4–5. **C.** Plasma glucose of mice injected with Pxx866. n=4–5. Results are expressed as mean ± SE. “*”, p< 0.05, vs. vehicle group. NS, not significant.

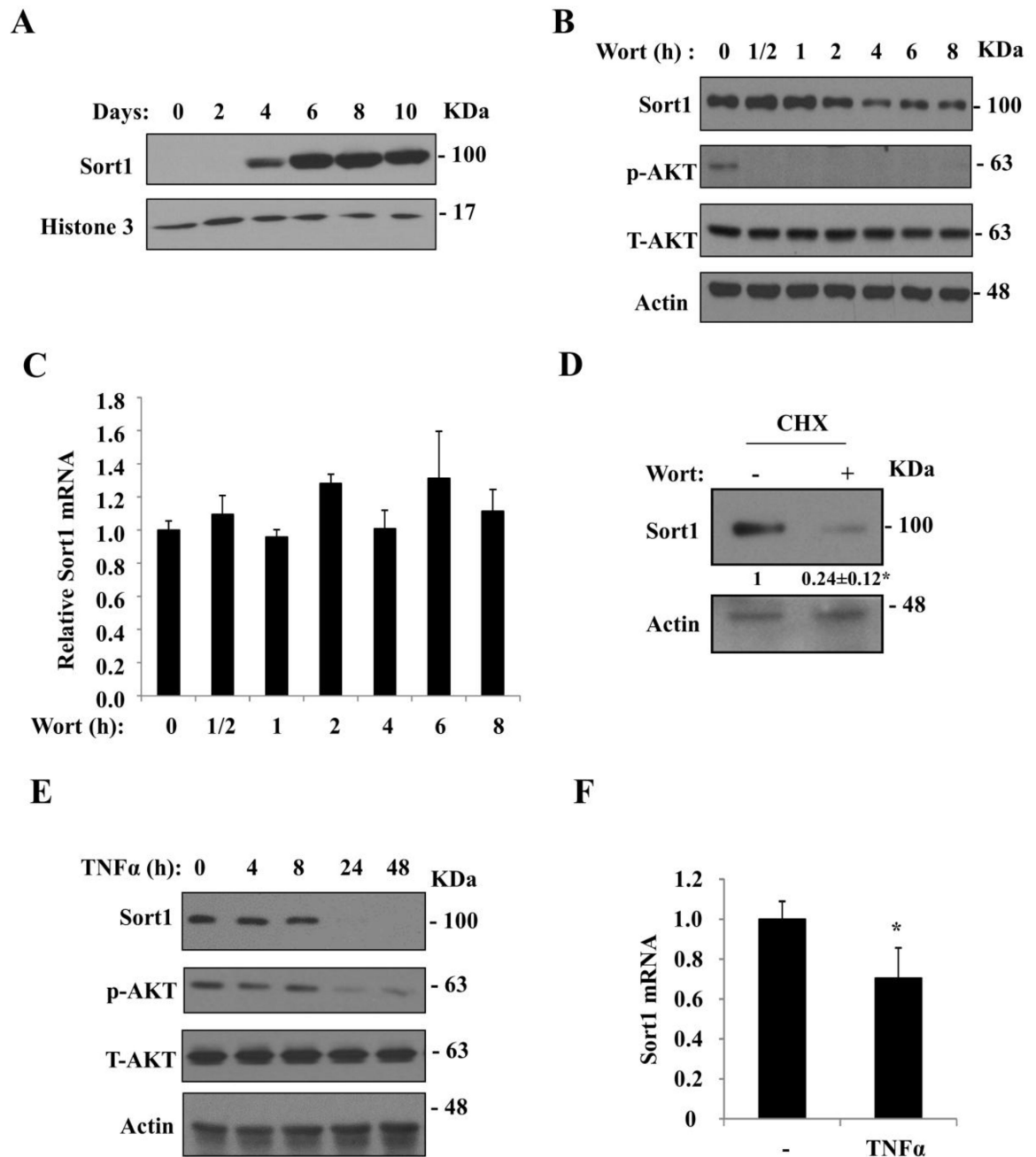


FIGURE 3. PI3K inhibitor and TNF α decreased Sort1 protein in 3T3-L1 adipocytes

A. Western blotting measurement of Sort1 protein expression during 3T3-L1 differentiation into adipocytes. **B, C.** Sort1 protein and mRNA in differentiated 3T3-L1 adipocytes cultured in insulin-containing maintenance medium and treated with 1 μ M wortmannin (Wort) in time course. Results of a representative real-time PCR assay was shown. Real-time PCR was done in triplicates and results are expressed as mean \pm SD. **D.** Differentiated 3T3-L1 adipocytes were cultured in maintenance medium and pre-treated with 100 μ g/ml cycloheximide (CHX) for 1 h, and then treated with 1 μ M wortmannin for 6 h. Sort1 protein was detected by Western blotting. A representative blot was shown. The average

densitometry value of 3 independent experiments was shown under the blot. **E, F**. Differentiated 3T3-L1 adipocytes were treated with 20 ng/ml mouse TNF α for time indicated. Culture medium containing TNF α was replaced every 24 h in cells treated for 48 h. **E**. Sort1 protein was detected by Western blotting. **F**. Sort1 mRNA was modestly reduced after 24 h TNF α treatment. Results are expressed as mean \pm SD. “*”, p< 0.05, vs. vehicle-treated controls. In **A, B, D** and **E**, anti-Sort1 antibody was used to detect Sort1 protein.

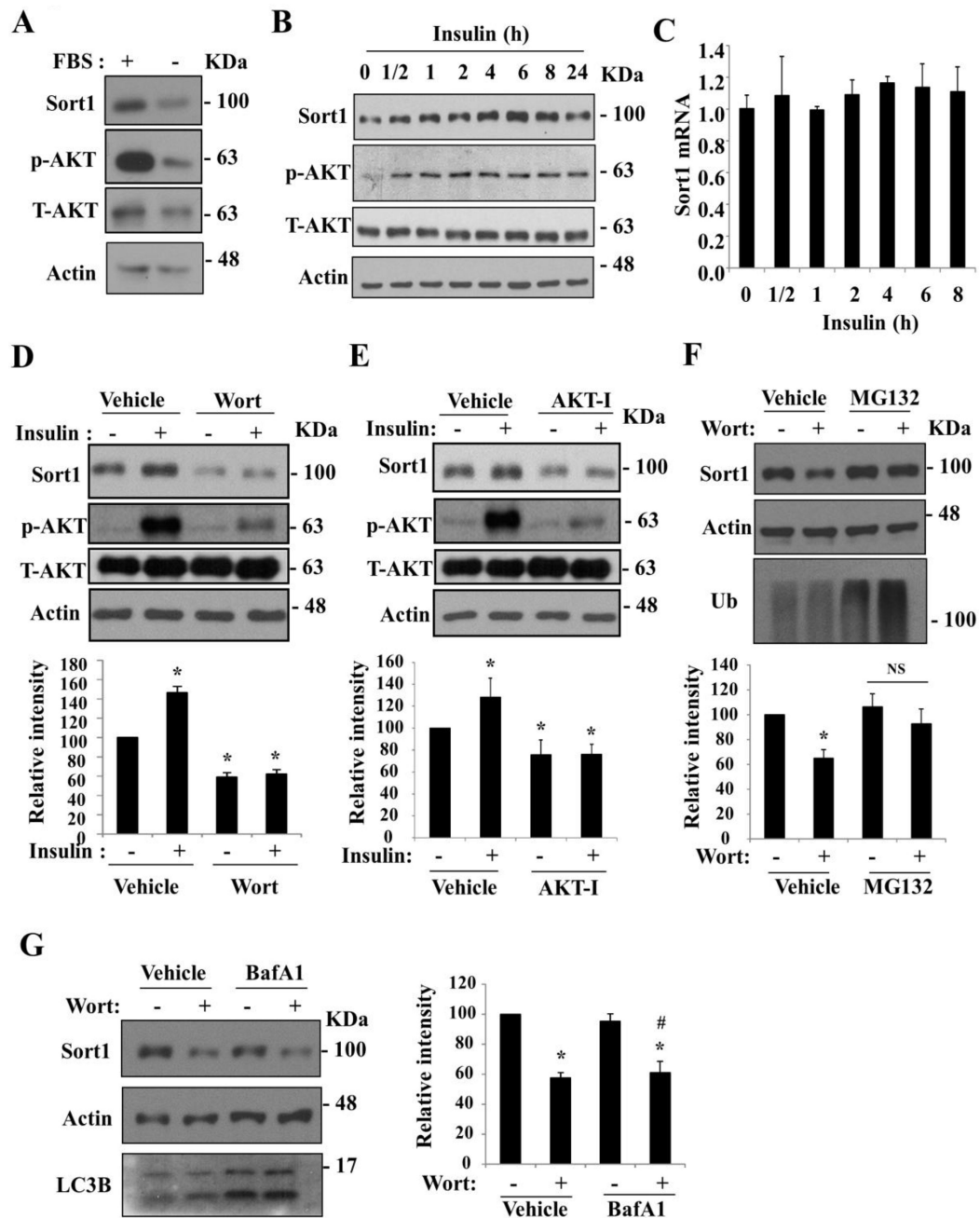


FIGURE 4. Insulin increased Sort1 protein in serum-starved 3T3-L1 adipocytes

A. Western blotting detection of Sort1 protein with anti-Sort1 antibody in differentiated 3T3-L1 adipocytes cultured in 10% FBS containing DMEM or FBS-free DMEM for 16 h. **B, C.** Detection of Sort1 protein or mRNA in differentiated 3T3-L1 adipocytes cultured in FBS-free DMEM for 16 h, and treated with 100 nM insulin in time course. Real-time PCR results of a representative assay were performed in triplicates and expressed as mean \pm SD. No significant changes were detected. **D, E.** Differentiated 3T3-L1 adipocytes were cultured in serum-free DMEM for 16 h, and then pre-treated with 1 μ M wortmannin (Wort) (**D**) or 10 μ M AKT inhibitor (AKT-I) (**E**) for 1 h before 100 nM insulin treatment for 6 h. Sort1

protein was detected by Western blotting with anti-Sort1 antibody. Vehicle: DMSO. Relative Sort1 band intensity of three independent experiments was expressed as bar graphs below the blot. “*”, vs. vehicle alone. **F, G**. Differentiated 3T3-L1 adipocytes were cultured in maintenance medium and were pre-treated with 10 μ M MG132 (**F**) or 10 nM Bafilomycin A1 (BafA1) (**G**) for 1 h before 1 μ M wortmannin (Wort) treatment for 2 h. Sort1 protein was detected by Western blotting with anti-Sort1 antibody. Relative Sort1 band intensity of three independent experiments was expressed as bar graphs below the blot. Ub. Ubiquitin antibody. “*”, vs. vehicle alone. “#”, vs. BafA1 alone.

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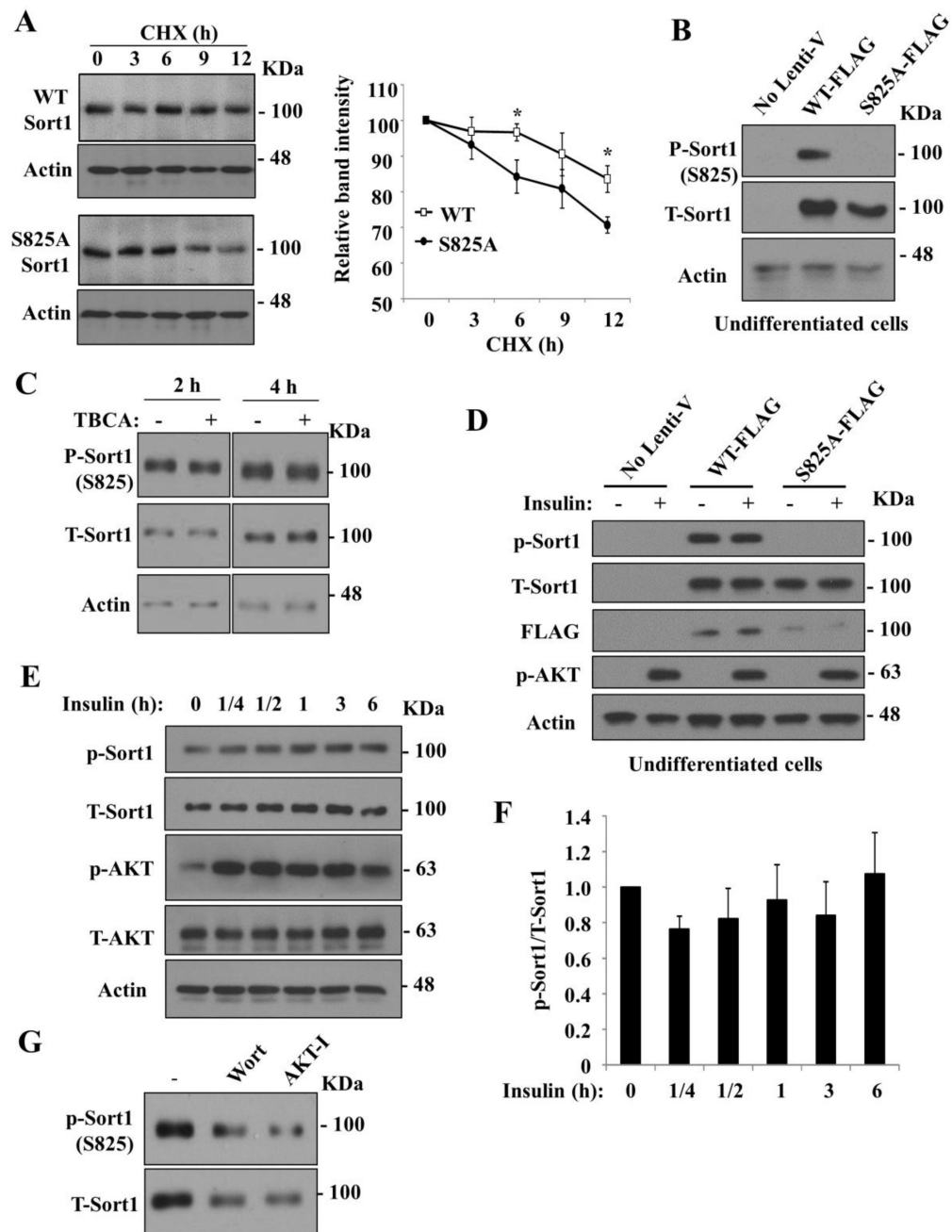


FIGURE 5. Phospho-Sort1 to total Sort1 ratio was not altered by insulin or PI3K inhibitor in 3T3-L1 adipocytes

A. Differentiated 3T3-L1 adipocytes stably expressing WT-Sort1-FLAG or S825A-Sort1-FLAG were cultured in maintenance medium and treated with 100 μ g/ml cycloheximide (CHX) for time indicated. Western blot was performed with anti-FLAG antibody. Right panel: Average Sort1 band intensity (not normalized to Actin band) of three independent experiments with 0 h set as 100. “*”, vs. WT at the same time point. **B.** Detection of phospho-Sort1 and total Sort1 with anti-Sort1 antibody in undifferentiated 3T3-L1 cells (no-Lenti-V) and undifferentiated 3T3-L1 cells stably expressing WT-Sort1-FLAG or S825A-

Sort1-FLAG. These cells were cultured in serum-containing medium. **C.** Detection of endogenous phospho-Sort1 and total Sort1 in differentiated 3T3-L1 adipocytes cultured in maintenance medium and treated with 10 μ M TBCA. Controls were treated with DMSO. **D.** Detection of phospho-Sort1 and total Sort1 in undifferentiated 3T3-L1 cells (no-Lenti-V) and undifferentiated 3T3-L1 cells stably expressing WT-Sort1-FLAG or S825A-Sort1-FLAG. Cells were serum-starved overnight. Some cells were then treated with 100 nM insulin for 1 h. Note: endogenous Sort1 protein was not detected in undifferentiated 3T3-L1 cells by anti-Sort1 antibody. **E, F.** Differentiated 3T3-L1 adipocytes were cultured in serum/insulin-free medium overnight, and then treated with 100 nM insulin in time course. Phospho-Sort1 and total Sort1 was measured by Western blotting. In F, the band intensity of phospho-Sort1 and total Sort1 was determined by ImageJ software. Results of 3 independent experiments were plotted as mean \pm SD. No significant changes of the p-Sort1/T-Sort1 ratio were detected. **G.** Phospho-Sort1 and total Sort1 protein was measured by Western blotting in differentiated 3T3-L1 adipocytes cultured in maintenance medium and treated with vehicle (DMSO), 1 μ M wortmannin (Wort) or 10 μ M AKT inhibitor (AKT-I) for 2 h.

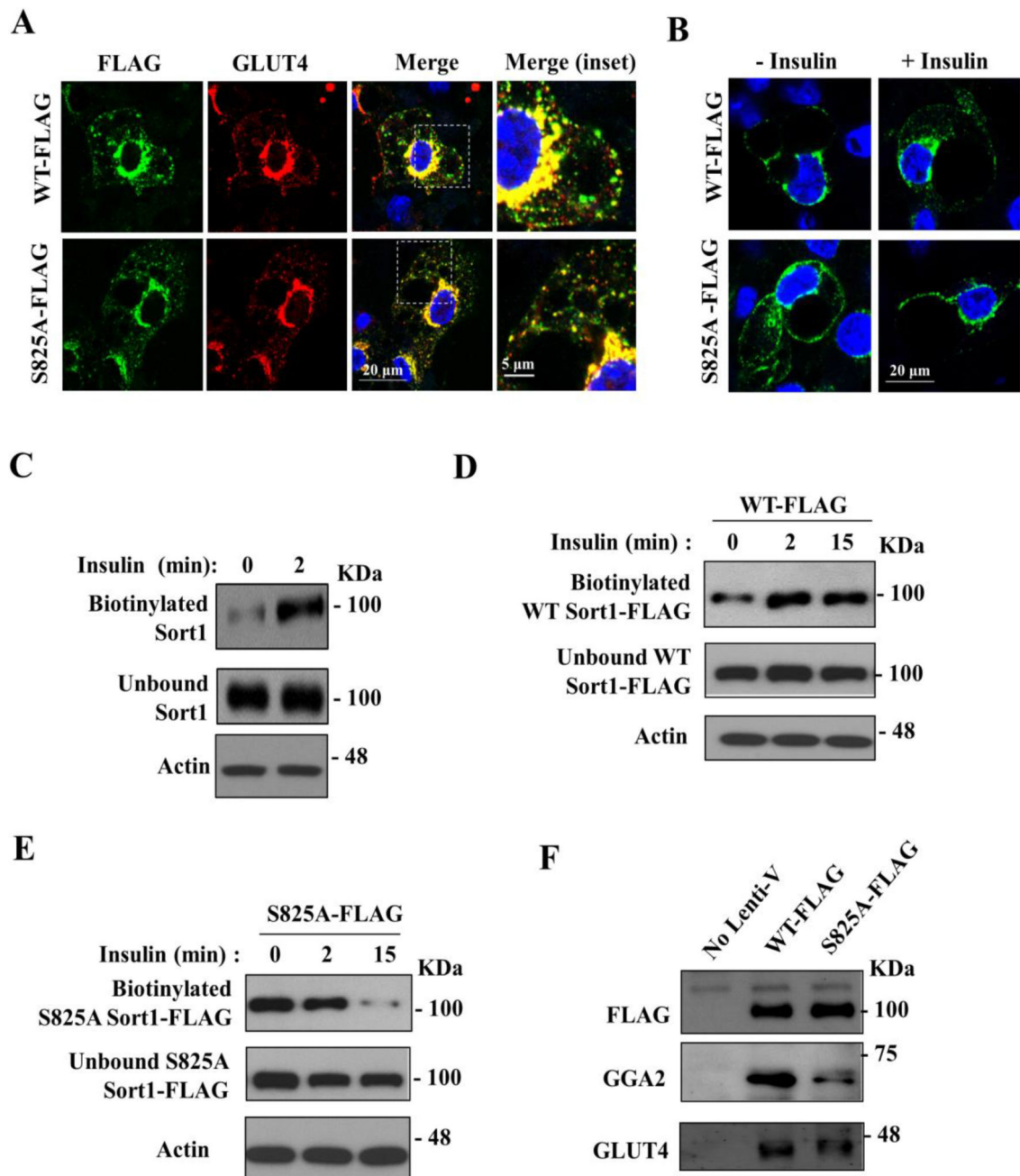


FIGURE 6. Abolishing S825 phosphorylation prevented insulin-regulated Sort1 membrane localization in 3T3-L1 adipocytes

A. Confocal microscope of differentiated 3T3-L1 adipocytes stably expressing WT-Sort1-FLAG or S825A-Sort1-FLAG. Cells were cultured in FBS-free medium for 16 h. Sort1-FLAG was stained with anti-FLAG antibody. Blue: DAPI staining of nuclei. **B.** Confocal microscope of differentiated 3T3-L1 adipocytes stably expressing WT-Sort1-FLAG or S825A-Sort1-FLAG. Cells were cultured in FBS-free medium for 16 h and then treated with 100 nM insulin for 15 min. Sort1 (green) was stained with anti-FLAG antibody. Nuclei were stained with DAPI (Blue). **C.** Differentiated 3T3-L1 adipocytes were cultured in serum-free

medium for 16 h, and treated with 100 nM insulin for 2 min. Surface biotinylation assays were performed. Endogenous biotinylated Sort1 and unbound Sort1 in the flow-through were detected with Sort1 antibody. **D, E.** Differentiated 3T3-L1 adipocytes expressing WT Sort1-FLAG or S825A Sort1-FLAG were cultured in serum-free medium for 16 h, then treated with 100 nM insulin for 2 and 15 min. Biotinylated Sort1-FLAG and unbound Sort1-FLAG in the flow-through were detected with anti-FLAG antibody. Actin in flow-through was also blotted. **F.** Differentiated 3T3-L1 adipocytes stably expressing WT-Sort1-FLAG or S825A-Sort1-FLAG were subjected to co-immunoprecipitation with anti-FLAG magnetic beads. Co-precipitated GGA2 and GLUT4 were measured by Western blotting. Differentiated adipocytes without stable Sort1-FLAG expression was subjected to immunoprecipitation as negative controls.