# Downregulation of *CPPED1* Expression Improves Glucose Metabolism In Vitro in Adipocytes

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We have previously demonstrated that the expression of calcineurin-like phosphoesterase domain containing 1 (*CPPED1*) decreases in adipose tissue (AT) after weight reduction. However, the function of CPPED1 in AT is unknown. Therefore, we investigated whether the change in *CPPED1* expression is connected to changes in adipocyte glucose metabolism. First, we confirmed that the expression of *CPPED1* decreased after weight loss in subcutaneous AT. Second, the expression of *CPPED1* did not change during adipocyte differentiation. Third, *CPPED1* knockdown with small interfering RNA increased expression of genes involved in glucose metabolism (adiponectin, adiponectin receptor 1, and *GLUT4*) and improved insulin-stimulated glucose uptake. To conclude, CPPED1 is a novel molecule involved in AT biology, and CPPED1 is involved in glucose uptake in adipocytes. ***Diabetes* 62:3747–3750, 2013**

Lifestyle modification improves glucose metabolism and results in a substantial reduction in the risk of type 2 diabetes in the long-term (1). In searching new putative genes related to obesity and type 2 diabetes, we have previously demonstrated a multitude of changes in adipose tissue (AT) gene expression in response to weight reduction in individuals with metabolic syndrome (2,3). Among the downregulated genes was calcineurin-like phosphoesterase domain containing 1 (*CPPED1*) (2); its function in AT is completely unknown.

Therefore, we continued to study the role of CPPED1 in AT in more detail. Interestingly, the experiment using a Simpson-Golabi-Behmel syndrome (SGBS) cell strain demonstrated an impact of *CPPED1* small interfering RNA (siRNA) on insulin-stimulated glucose uptake in mature adipocytes. Overall, the results demonstrate that CPPED1 is a novel molecule expressed in AT and is related to adipocyte function.

## RESEARCH DESIGN AND METHODS

The design of the Gene Expression in Obesity and Insulin Resistance (GENOBIN) study has been reported earlier (2,3). Altogether, 46 overweight or obese (BMI 28–40 kg/m$^2$) subjects 40–70 years of age were randomly assigned to one of two groups: a weight-reduction group ($n$ = 28) or a weight-maintenance control group ($n$ = 18) (2). After an overnight fast, AT samples were taken by needle biopsy from subcutaneous AT before and after the intervention (8 months) under local anesthesia (lidocaine 10 mg/mL without adrenaline).

The GENOBIN study was performed in accordance with the standards of the Helsinki Declaration, and the ethics committee of the District Hospital Region of Northern Savo approved the study plans. All participants gave a written informed consent.

**SGBS cell strain.** Human preadipocyte cell strain SGBS is characterized by a high capacity for adipogenic differentiation (4). The cells were cultured as described previously (5).

**Knockdown of the expression of the *CPPED1* gene in SGBS cells.** RNA interference was used for knocking down the expression of the *CPPED1* gene in mature adipocytes. ON-TARGETplus SMARTpool siRNA for *CPPED1* was purchased from Dharmacon (Thermo Scientific, Lafayette, CO). There are four target siRNA sequences included in the pool: AGAAAAUUGUUCACCGAUA, UAAAUGCACUAAUGCGAAA, CGGAGGACCUGAAGCGAGU, and CCUUUAAAAUGGAGCGAAU. The negative control for the siRNA (scrambled) used in the experiment was Allstars negative control siRNA (Qiagen, Valencia, CA). The siRNA was transfected into the cell by using HiPerFect transfection reagent (Qiagen) according to the instructions.

In brief, the SGBS cells were cultured in 12-well plates and induced into mature adipocytes. On day 14 of differentiation, the medium was replaced with Dulbecco's modified Eagle's medium/Ham's F12 nutrient mixture (1:1) supplemented with 33 μmol/L biotin, 17 μmol/L pantothenate, 10 μg/mL transferrin, and 20 nmol/L insulin. The cells were transfected with 50 nmol/L siRNA and incubated for the indicated time points. Knockdown of *CPPED1* expression was confirmed by reverse-transcriptase quantitative PCR (RT-qPCR) and Western blot. The effect of *CPPED1* knockdown on high-molecular-weight (HMW) adiponectin secretion into the conditioned medium was measured with a commercial ELISA kit purchased from Millipore (St. Charles, MO) according to the manufacturer's protocol.

**Insulin-stimulated glucose uptake in SGBS cells.** The SGBS cells were cultured in 12-well plates and induced into mature adipocytes. On day 17 of differentiation, the cells were washed twice with PBS and preincubated with KRH buffer (20 mmol/L HEPES [pH 7.4], 118 mmol/L NaCl, 4.8 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$) for 2 h at +37°C. After preincubation, the cells were incubated in the presence of 100 nmol/L wortmannin for 30 min (when indicated) and followed by incubation with 1 μmol/L insulin for 20 min. Next, 0.5 μCi/mL labeled 2-deoxy-D-[$^3$H] glucose (Amersham TRK672; GE Healthcare, Buckinghamshire, U.K.) and 0.2 mmol/L D-glucose were added for an additional 15 min at +37°C. The reaction was terminated by placing the cells onto the ice and washing three times with ice-cold PBS.

The cells were solubilized with 200 μL of 0.2 N NaOH per well and incubated 1.5 h at room temperature with constant shaking. The cell lysate (100 μL) was transferred to a 2.0-mL Eppendorf tube, and scintillation liquid was added for radioactivity counting. Glucose uptake was normalized to protein content as measured from the remaining cell lysate using the Bio-Rad protein assay (DC Protein Assay; Bio-Rad, Hercules, CA). Protein concentrations were measured according to the manufacturer's instructions (DC Protein Assay) using Wallac 1420.

**RNA extraction, cDNA synthesis, and RT-qPCR.** Total RNA extraction and cDNA synthesis of AT samples have been described previously (2). For cultured SGBS cells, the RNeasy Mini Kit was used for the total RNA extraction (Qiagen, Valencia, CA) and iScript cDNA Synthesis Kit (Bio-Rad) according to instructions provided by the manufacturer.

RT-qPCR with TaqMan chemistry (Applied Biosystems) using an ABI Prism 7500 analyzer (Applied Biosystems) was used. The analysis for the relative quantity of a specific gene before and after the intervention in AT of the GENOBIN was performed as described previously (2). The expression of target genes was normalized to cyclophilin A1 (PPIA) expression for AT samples and SGBS cells. Expression of the target genes in cultured SGBS cells was normalized to the endogenous control using the formula $2^{-ddCt}$ (6).

***Western blot.*** For Western blot, cells were rinsed twice with PBS and then lysed in RIPA Lysis and Extraction Buffer (Pierce, Rockford, IL) freshly

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supplemented with 1 mmol/L EDTA, 1 mmol/L PMSF, 1 mmol/L $Na_3VO_4$, 1 mmol/L NaF, and proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), followed by centrifugation (13,000 rpm, 15 min, +4°C) and removal of the soluble fraction. The protein concentration was determined using the Bio-Rad protein assay (DC Protein Assay). Equal amounts of protein were separated using SDS-PAGE and transferred to the polyvinylidene fluoride (PVDF) membrane (GE Healthcare).

Rabbit anti-human CPPED1 and adiponectin (ADIPOQ) were used as primary antibodies (Sigma-Aldrich). The primary antibodies were detected with goat anti-rabbit peroxidase-conjugated secondary antibody (Pierce). Signals were detected using Amersham Advance Western Blotting Detection Kit (GE Healthcare) and ImageQuant Capture-RT ECL for Windows version 1.0.1 (GE Healthcare). Densitometric analysis was performed by ImageJ version 1.45s (ImageJ; National Institutes of Health, Bethesda, MD). A rabbit anti-human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam) was used for normalization.

**Statistical analyses.** The gene expression data of GENOBIN was analyzed using the SPSS software for Windows version 14.0 (SPSS Inc., Chicago, IL), and data are provided as mean ± SD, unless otherwise indicated. Paired-samples Student *t* test was used for comparing the baseline and end point measurements within the study group. A value of $P < 0.05$ was considered statistically significant.

All results of the SGBS cell culture studies were analyzed using the GraphPad Prism5 software for Windows version 5.03 (GraphPad Software, San Diego, CA), and results are expressed as mean ± SEM. Statistical significance was determined with independent-samples Student *t* test or one-way ANOVA with Bonferroni multiple comparison test (indicated in figures). A value of $P < 0.05$ was considered statistically significant.

## RESULTS

**GENOBIN study.** A previously published reduction in *CPPED1* expression in AT after weight reduction using Affymetrix microarrays (2) was confirmed with RT-qPCR (Fig. 1).

***CPPED1* expression during adipocyte differentiation.** The expression of *CPPED1* mRNA was at a maximal level in preadipocytes and did not change during the differentiation process (Fig. 2*A*), whereas peroxisome proliferator–activated receptor γ 2 was upregulated during SGBS differentiation, as expected (Fig. 2*B*).

***CPPED1* knockdown leads to increased insulin-stimulated glucose uptake.** The role of CPPED1 in adipocytes was studied by siRNA in SGBS adipocytes. As shown in Fig. 3*A* and *B*, the knockdown of *CPPED1* for 48 h decreased the expression of *CPPED1* at both the mRNA and protein levels. The *CPPED1* knockdown led to increased mRNA expression of *ADIPOQ*, adiponectin receptor 1, and *GLUT4*, and to decreased mRNA expression of *GLUT1* and leptin (*LEP*) (Fig. 3*B*).

The effects of *CPPED1* siRNA on the expression of genes related to glucose metabolism suggested a significant role of CPPED1 in adipocyte metabolism. Therefore, we next investigated basal and insulin-stimulated glucose uptake in cells treated with scrambled and *CPPED1* siRNA. Insulin-stimulated glucose uptake increased in *CPPED1* siRNA–treated cells by +74% ($P < 0.05$) compared with control cells (Fig. 3*C*). Wortmannin treatment abolished the increase in insulin-stimulated glucose uptake in both conditions.

Finally, the protein expression of ADIPOQ increased time dependently (Fig. 3*D*), leading to a significant increase in ADIPOQ protein at 96 h after *CPPED1* siRNA treatment (+32%, $P < 0.05$). In line with this, the reduction of *CPPED1* expression for 48 h tended to increase HMW adiponectin secretion ($P = 0.057$) into the conditioned medium (Fig. 3*E*). The protein expression of GLUT4 did not change after *CPPED1* siRNA treatment (Fig. 3*F*).

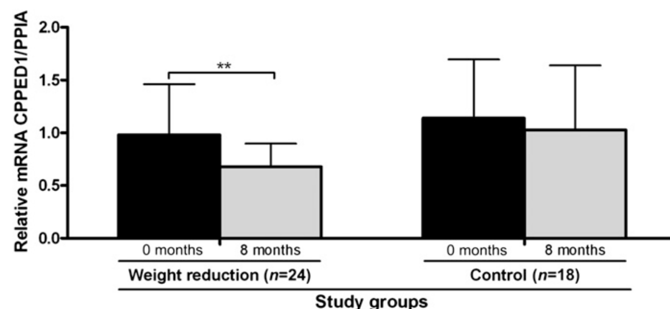


**FIG. 1. The relative mRNA expression of *CPPED1* in subcutaneous AT samples at baseline (black bars) and after the intervention (gray bars) in weight-reduction group and weight-maintenance control group. The values are expressed as relative gene expression levels normalized to endogenous control PPIA. $P < 0.05$ was considered statistically significant (comparisons were made within groups). The final number of patients included in the data analysis is indicated in the figure. **$P < 0.01$.**

## DISCUSSION

We suggest that the decrease in mRNA expression of *CPPED1* in AT after weight loss in humans may have important implications because we now demonstrate that downregulation of *CPPED1* by siRNA leads to an increased insulin-stimulated glucose uptake in cultured mature adipocytes possibly via adiponectin-mediated mechanisms.

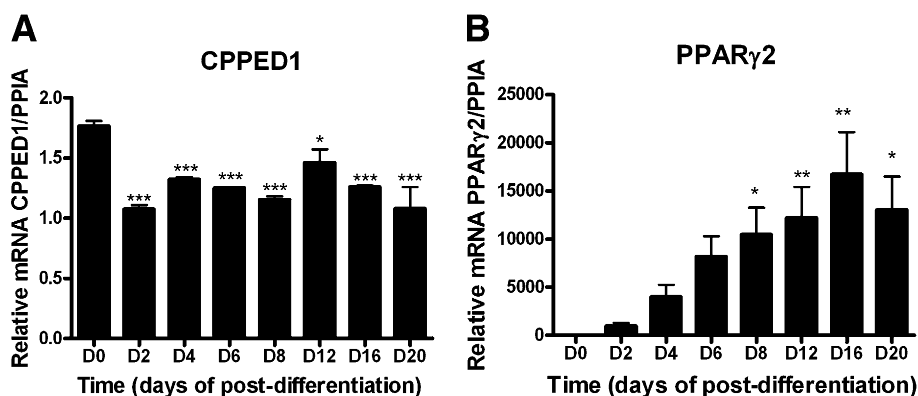


**FIG. 2. *CPPED1* (*A*) and *PPARγ2* (*B*) mRNA expressions in cultured SGBS cells during adipocyte differentiation. The SGBS cells were induced to differentiate and were harvested in different time points during adipocyte differentiation. Statistical significance was tested using one-way ANOVA with Bonferroni multiple comparison test. The graph shows the means ± SEM from four independent experiments, and the values are expressed as relative gene expression levels normalized to endogenous control *PPIA*. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (indicated time point vs. day [D] 0).**

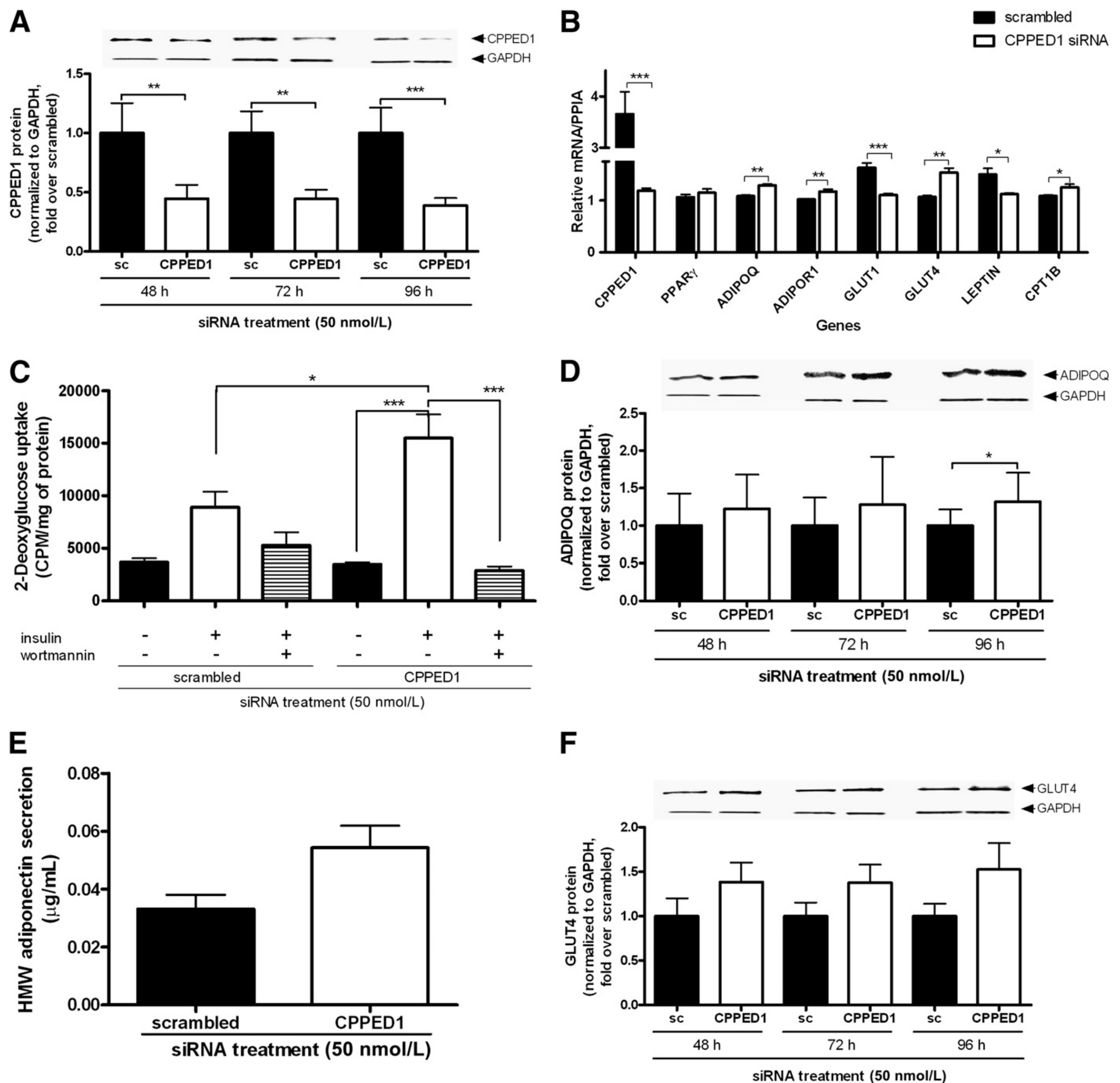


**FIG. 3.** ***A***: **A time-course effect of *CPPED1* knockdown on the protein expression of CPPED1. The values for the protein experiments are normalized to GAPDH. Statistical significance was tested using independent-samples Student *t* test. *B*: The effect of *CPPED1* knockdown on the mRNA expressions of selected genes. The values for the gene expression data are expressed as relative gene expression levels normalized to endogenous control *PPIA*. Statistical significance was tested using independent-samples Student *t* test. *C*: The effect of *CPPED1* knockdown for 48 h on insulin-stimulated glucose uptake (the values are expressed as counts per minute [CPM] normalized to the protein amount [mg] of corresponding well). Statistical significance was tested using one-way ANOVA with Bonferroni multiple comparison test. *D*: A time-course effect of *CPPED1* knockdown on the protein expression of ADIPOQ. The values for the protein experiments are normalized to GAPDH. Statistical significance was tested using independent-samples Student *t* test. *E*: The effect of *CPPED1* knockdown for 48 h on the secretion of HMW adiponectin into the conditioned medium. The graph shows the means ± SEM and/or representative figures within time of siRNA exposure from at least three independent experiments. *F*: A time-course effect of *CPPED1* knockdown on the protein expression of GLUT4. The values for the protein experiments are normalized to GAPDH. Statistical significance was tested using independent-samples Student *t* test. *$P$ < 0.05; **$P$ < 0.01; ***$P$ < 0.001. sc: scrambled.**

Collectively, these results provide new data that CPPED1 is involved in glucose metabolism in AT, and could be a novel gene related to AT dysfunction in obesity.

The in vitro experiments in SGBS cells showed that the *CPPED1* mRNA expression was not changed during adipocyte differentiation whereas peroxisome proliferator–activated receptor $\gamma$ 2 was upregulated as expected, indicating that *CPPED1* is not directly regulated in the adipocyte differentiation process. In addition to adipocytes, AT is composed of stromal vascular fraction cells, including preadipocytes, endothelial cells, and several immune cells (7,8). Thus, we cannot distinguish which other cell types are contributing to the expression of CPPED1 in AT and further studies are needed. However, our results

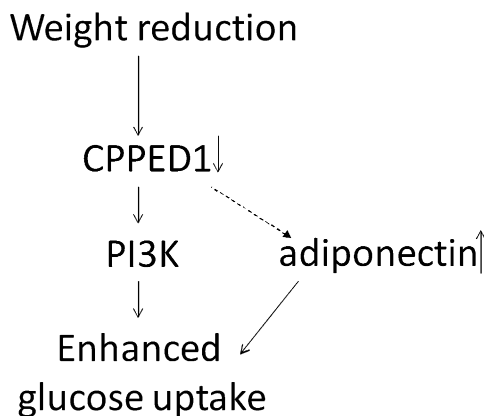


**FIG. 4. A suggested model for the role of CPPED1 in glucose metabolism. We hypothesize that the improvement in glucose uptake after weight loss could be partly due to the decreased expression of CPPED1, and possibly adiponectin signaling could either directly or indirectly play a role in this regulation. Intact arrows, known effects/effects in cell experiments; dashed arrows, possible interactions; ↑, upregulation; ↓, downregulation.**

show that at least preadipocytes and mature adipocytes are involved in CPPED1 expression.

To better understand the role of CPPED1 in adipocytes, we studied the effect of *CPPED1* knockdown by siRNA in SGBS adipocytes on the expression of genes involved in adipogenesis and glucose metabolism. The downregulation of *CPPED1* mRNA enhanced the insulin-stimulated glucose uptake in mature adipocytes, suggesting that CPPED1 is involved in glucose metabolism in adipocytes. To study the possible involvement of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in this process, the cells were incubated with the PI3K-specific inhibitor wortmannin. It turned out that wortmannin significantly decreased the *CPPED1* knockdown–induced improvement in glucose uptake, suggesting that the effect of *CPPED1* downregulation by siRNA on insulin-stimulated glucose uptake may be mediated through a PI3K/Akt-dependent pathway. The role of CPPED1 in insulin signaling in adipocytes needs to be elucidated in more detail, including experiments in which CPEDD1 is overexpressed in different cell types.

Interestingly, we also found that *CPPED1* may regulate adiponectin signaling in AT. The knockdown of *CPPED1* expression in mature adipocytes increased the gene and protein expression of ADIPOQ, and there was also a clear trend for enhanced secretion of HMW adiponectin into the conditioned medium. Furthermore, gene expression levels of adiponectin receptor 1 and *GLUT4* were increased due to *CPPED1* knockdown. In humans, adiponectin levels are known to be inversely related to obesity, insulin resistance, and type 2 diabetes (9). Moreover, adiponectin has been shown to modify glucose metabolism (10) by improving insulin sensitivity and promoting glucose uptake (9,11) through adiponectin receptors 1 and 2 (10,12). Therefore, we propose a model (Fig. 4) that *CPPED1* knockdown–induced improvement in insulin-stimulated glucose uptake could be due to adiponectin-mediated mechanisms.

In conclusion, knockdown of *CPPED1* expression enhances insulin-stimulated glucose uptake in mature adipocytes, leading to improved glucose metabolism possibly via adiponectin-mediated mechanisms. The potential of CPPED1 knockdown in the treatment of obesity-related phenotypes needs to be investigated further.

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No potential conflicts of interest relevant to this article were reported.

M.V. performed the experiments, researched data, and wrote the manuscript. D.K., P.K., and M.E. contributed to coordination and edited the manuscript. M.K. participated in the study design and coordination and edited the manuscript. J.P. contributed to coordination of the manuscript and edited the manuscript. M.U. contributed to coordination of the manuscript, edited the manuscript, supervised the study, and participated in the study design. L.P. supervised the study, participated in the study design, and edited the manuscript. M.U. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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