KCTD15 Gene Expression Regulates Adipocyte Differentiation and Is Associated with Obesity in Humans

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Abstract

Background It has been substantiated by Genome-wide association studies (GWAS) that genetic disparity influences the fat levels and obesity risk in various populations.

Objective A meta-analysis of 15 genome-wide association studies identified regularly-used obesity-related variables at eight gene loci, inclusive of FTO, MC4R, TMEM18, KCTD15, GNPDA2, SH2B1, MTCH2 and NEGR1. However, the expression profiles of eight obesity-related genes remain largely unknown.

Design, Patients, Measurements Based on the fundamental propositions specified in the Declaration of Helsinki, adipose tissue specimens were collected from 23 obese and 16 normal weight subjects, and the expression profiles of eight obesity-related genes identified by GWAS were examined in these white adipose tissues (WAT). The effects of KCTD15 gene on adipocyte differentiation in mature 3T3-L1 adipocytes were assessed.

Results Among those obesity-related genes identified by GWAS, SH2B1, FTO, TMEM18 and KCTD15 are expressed at lower level in WAT of obese subjects compared to normal weight subjects, while NEGR1 shows higher expression level in the obese subjects. No changes were detected for MTCH2, GNPDA2 and MC4R. The expression of KCTD15 was negatively correlated with Body mass index (BMI) and abdominal circumference. The siRNA-mediated knockdown of KCTD15 significantly suppressed cellular lipid accumulation during adipocyte differentiation.

Conclusion This is the first study to identify the expression patterns of eight obesity-related genes bearing association with being obese in white adipose tissue of overweight people in China. Our study demonstrates that the expression of KCTD15 is associated with obesity in humans. Meanwhile, KCTD15 plays a critical role in the regulation of adipogenesis.

Background

Obesity is a result of excessive amount of triglycerides storage in adipose tissue, which is the result from a significantly higher amount of calories acquired than that being consumed by human body. White adipose tissue (WAT) is accepted as essential for the regulation energy homeostasis ⁽¹⁾, and the aberrantly increased mass of WAT is a phenotype of obesity that is determined by adipocyte number (adipocyte differentiation) and size (fat storage) ⁽²⁾. Studies on the expression profiles of obesity-related genes in adipose tissue have provided valuable information for understanding the development and regulation of obesity ⁽³⁾. A solid proof is presented by the Genome-wide association studies (GWAS) that candidate obesity-related genes influence the fat level and obesity risk in various populations. An updated map of the human obesity gene published by Rankinen et al, which has become a significant instrument for researchers in this respect ⁽⁴⁾. In addition, A meta-analysis of 15 genome-wide association studies further identified regularly-used obesity-related variables at eight gene loci, inclusive of Fat Mass and Obesity-associated protein (FTO), melanocortin 4 receptor (MC4R), transmembraneprotein18 (TMEM18),

potassium channel tetramerization domain containing 15 (KCTD15), glucosamine-6-phosphate isomerase 2 (GNPDA2), SH2B adapter protein 1 (SH2B1), mitochondrial carrier homolog 2 (MTCH2) and neuronal growth regulator 1 (NEGR1) ⁽⁵⁾. FTO is the earliest that was found to contribute to non-syndromic overweight among people ⁽⁶⁾. SH2B1 and MC4R were proven to be associated with the regulation of body weight through knockout experiments ^(7, 8). TMEM18 and NEGR1 are likely to have a link either with appetite and energy balance via its central sites of action or via an influence on adipogenesis in the periphery ⁽⁹⁾. KCTD15 seems likely to function as an energy balance regulator ⁽¹⁰⁾. GNPDA2 is associated with hexosamine signaling pathway, causing HFD (high fat diet)-fed rats to exhibit the characteristics of obesity and insulin-resistant phenotype. SH2B1 promotes adipocyte differentiation. Many studies have reported the expression of these genes in adipose tissue from high fat diet (HFD) fed or fasted mice, diet-induced obese rats and Zucker diabetic fatty (ZDF) rats (10–13); however, in-depth research is considered necessary to delineate the underlying process for these genes to regulate energy homeostasis and obesity.

Throughout this research, an investigation is conducted into how these eight-candidate obesity-related genes are expressed in WAT from normal weight and obese subjects with the correlation between the expression of these genes and BMI to identify the genes that were significantly associated with obesity analyzed for further study. Interestingly, we found that KCTD15 showed the strongest correlation with BMI. KCTD15 was first identified in human as a BTB domain-containing protein with a conserved C-terminus (14), but the function of this protein remains unelucidated. Here, we used the mouse-derived 3T3-L1 pre-adipocyte cell line as an in vitro adipocyte differentiation model for the purpose of studying the function of KCTD15 during adipocyte differentiation (15). The role of KCTD15 in adipogenesis was determined by examining its expression profile during 3T3-L1 differentiation. Additionally, the importance of KCTD15 during adipocyte differentiation and lipid accumulation was also further determined by using siRNA-mediated knockdown approaches.

Method

Subject tissue samples

Based on the fundamental propositions laid out in the Declaration of Helsinki, we were granted permission from the adult subjects or guardians on behalf of the minors participants involved in our study by written before sample collection. Adipose tissue specimens were obtained from 6 overweight subjects, 17 obese subjects (14 females, 9 males) and 16 normal weight subjects (13 females, 3 males) (aged from 11 to 79 years, with a median of 45 years) who had an elective surgery at Xinqiao Hospital of Third Military Medical University, Chongqing, China. Demographic data of the studied subjects is shown in Supplementary Table S1, S2. All subjects, who were neither smoker nor under lipid lowering medications, went through thorough clinical phenotyping at an accelerated rate (12 h, overnight). To calculate the BMI (weight/height squared) (kg/m²), a measurement was taken of the weight (kg) of the subjects who was wearing cloth that weighed as little as 0.1 kg. Besides, the subjects' height (bare foot) was determined using a stadiometer (cm). On the other hand, the distance around their abdomen was

ascertained utilizing a measuring tape. In order to figure out how prevalent overweight and obesity was, a calculation was performed with a combination of the body mass index (BMI) (normal weight 18.5-23.9 kg/m²; overweight 24-27.9 kg/m²; obesity ≥ 28 kg/m²) and the explanation of abdominal circumference (abdominal obesity: male ≥ 90 cm, female ≥ 85 cm) provided by Working Group on Obesity in China (16). Those subjects with cancer, HIV, collagen diseases and syphilis were excluded from the study. We received clearance granted by the studied participants. The project was granted approval from the local ethics committee of Third Military Medical University. Before the isolation of total RNA and protein, sampling of latest adipose tissue was conducted intraoperatively and immediately for freezing in liquid nitrogen and storage at -80 °C.

RNA isolation and RT-PCR

Human WAT with TRIzol® Reagent (Invitrogen) was involved for the extraction of total RNA and one microgram of total RNA was applied to synthesize cDNA by using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, K1611) in line with the requirements specified by the manufacturer, which was followed by PCR amplification with specific forward and reverse primers for 35 to 45 cycles. To enable internal control, GAPDH was utilized. The specific primers were used for the human genes are listed (Supplementary Table S4). The mouse primers of KCTD15 used in the study are as follows:

forward, 5'-ATGGGGAGATTTTCCGCTAC,

reverse, 5'-GGGGCTCCTGCTTTATCC.

A separation of the PCR products was conducted in 3% agarose gels for a subsequent visualization under UV light. Then, a quantitative analysis was performed to determine the density of the band using Quantity One software (Bio-Rad). The relative expression of all the genes detected were normalized to the GAPDH or β -actin.

Cell growing under controlled conditions culture and induction of differentiated adipocyte

While sustaining 3T3-L1 pre-adipocytes, the standard protocol was applied to facilitate them to differentiate (17). Briefly, Previous to confluence, Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% newborn calf serum (SH30401.01, Hyclone) and 1% antibiotics (Penicillin/Streptomycin/Amphotericin B) was involved to grow 3T3-L1 pre-adipocytes (we named the cell confluence day as Day-2, D-2). With 48 hours past cell confluence (Day 0, D0), the cells were induced to differentiation by exposing to differentiation medium containing 1 μ mol/L dexamethasone (D2915, Sigma), 0.5 mmol/L isobutyl methylxanthine (I5879, Sigma), 2 μ mol/L rosiglitazone (R2408, Sigma), 10 μ g/mL insulin (I6634, Sigma), and 10% fetal bovine serum (FBS; A15-151, PAA) for three days. When it approached the end of the 3rd day, DMEM supplemented only with 10 μ g/mL insulin and 10% FBS was used as a substitute of the original substance to grow the cells, and was subjected to replenishment for every two days. Upon the completion of the process to differentiate, a minimum of 90% of the cells accumulated lipid droplets at Day 10, for which they were selected as mature adipocytes.

siRNA transfection

The siRNA transfection of 3T3-L1 pre-adipocytes was performed as indicated (18). GenePharma was applied to the design and synthesis of mouse KCTD15 siRNA (Shanghai, China). The siRNA sequences are indicated here:

Mouse KCTD15 siRNA #1 5'-GACGUCUCAAACUGCUGCUU,

#2 5'-CAACUGCUGUCCGGAUAAA,

#3 5'-GGCUACUGUAGACUCAACU;

Negative control siRNA, 5'-UUCUCCGAACGUGUCACGU.

3T3-L1 preadipocytes were grown to 30–50% confluency in 35 mm dishes and transfected with siRNA oligonucleotides by applying Lipofectamine™ RNAiMAX (Invitrogen, 13778-150) according to the instructions of the manufacturer. Forty-eight hours later, the knockdown efficiency was detected by RT-PCR to facilitate differentiation, the standard differentiation protocol was applied.

Oil Red O dyeing

Subsequent to obtaining the mature adipocytes by in vitro differentiation, the lipid droplets in the mature adipocyte cells were monitored by adopting the approach of modified Oil Red O staining (19). In brief, the cells were washed twice with PBS before fixed with 10% formaldehyde for a duration of 1h at room temperature. Afterwards, The cells were washed twice with 60% isopropanol and then dyed by applying Oil Red O solution for 1 h. Images of the Oil Red O-stained cells were derived from using with Leica DMI3000 B inverted microscope.

Triglyceride assay

Triglycerides can be quantified with the triglyceride assay kit E1003-2 (Applygen Technologies Inc, Beijing, China). Briefly, cells ended up being harvested by trypsinization, rinsed three times with PBS and then lysed in lysis buffer. The supernatant was assayed in line with the requirements laid out by the manufacturer.

Western blotting

Total protein lysate was extracted from 3T3-L1 cells at different time points during differentiation with RIPA buffer (20 mM Tris-HCl, pH7.4, 20% glycerol, 0.5 % NP40, 1 mM MgCl₂, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA.). Protein concentrations were measured using the Bradford Assay, and 100 µg protein extracts were subjected to SDS-PAGE. Then proteins were transferred to a PVDF membrane, blocked with 5% non-fat milk and incubated with first antibodies (anti-KCTD15, Abcam ab106373, 1: 1000; anti-β-Tubulin, Sigma t-5293, 1: 10000) for 1 h at R.T. After incubation with secondary antibody against mouse

(Dakocytomation, p0161, 1:10,000) or rabbit (Dakocytomation, p0448, 1: 10,000) for 1 h at R.T, the membrane was visualized by ECL Western Blotting Detection Kit (RNP2232, GE healthcare).

Statistical Analysis

All statistics were interpreted as the mean ± SEM. The correlation coefficient, Student’s t-test (for single comparison) and one-way ANOVA (for group comparisons) were involved to analyze data where appropriate using SPSS 16.0 for Windows (SPSS, part of IBM Corporation; Somers, NY). Tukey’s post hoc test was applied under the circumstance of a necessary comparison among individual groups. *P* < 0.05 was regarded as being of statistical importance.

Ethical Aspects

Permission was granted for this research by the Human Research Ethics of Xinqiao Hospital of Third Military Medical University, under the number of ChiCTR-RNRC-11001441, and all parents signed the informed consent form.

Results

Clinical characteristics of the study subjects

Body mass index (BMI) is a prevalent indicator to rate individuals as normal weight or obese. In China, obesity is defined as an individual with BMI of 28 kg/m² and above, and this number has been used as a guideline on preventing people from getting obese or overweight (20). To date, eight genes have already been widely accepted as associated with obesity according to the genome-wide association studies. To further explore the relationship between these obesity-related genes and obesity, adipose tissue specimens from 23 obese subjects and 16 normal weight subjects were collected. The clinical characteristics data of these subjects are listed in Table1. Compared with normal subjects, who had an average BMI of 21.06±1.79 and abdominal circumference of 74.13±4.47, The obese subjects had a significantly higher average BMI of 28.78±1.78 and abdominal circumference of 105.6±6.07.

Table 1. Demographic and clinical characteristics of the study subjects. Data presented as the mean±standard deviation. * *P* < 0.05 was considered significant.

Indexs	Cases(n=23)	Control(n=16)	P
BMI (kg/m ²)	28.78±1.78*	21.06±1.79	< 0.0001
Abdominal Circumference(cm)	105.6± 6.07*	74.13± 4.47	< 0.0001

The expression of eight obesity-related genes in human adipose tissue and the correlation of these genes with BMI

To detect how the eight obesity-related genes are expressed at RNA level, total RNA was isolated from adipose tissues of both normal weight and obese subjects. RNA was later amplified by RT-PCR, and the representative PCR results are shown in Fig.1A. PCR products were quantified with Quantity One software (BioRad) and the relative expression of each gene was normalized to GAPDH. As indicated by Fig.1B, the expression of KCTD15 and SH2B1 were substantially significant among the obese individuals relative to the individuals with a normal weight, whereas the expression of TMEM18 and FTO was slightly less significant in the obese individuals than with normal people. In comparison, the expression of NEGR1 increased significantly among those obese subjects ($P=0.0092$). Nonetheless, there is no notable variation with regard to gene expressions of the MTCH2, GNPDA2 and MC4R between these two groups.

An investigation was carried out into how the seven genes are expressed in both standard weight and obese individuals and determined whether these genes are linked to obesity by analyzing the correlation coefficient for gene expression and BMI. Those variables with P values less than 0.05 were considered to be correlated. Thus, TMEM18, SH2B1, FTO and KCTD15 were negatively correlated with BMI, while NEGR1 was positively correlated with BMI (Fig.1C). The representative correlation curve for KCTD15 showed the most significant negative correlation with BMI (Fig.1D) and correlation with abdominal circumference (Supplement TableS3). Among these genes, SH2B1 has been reported to promote adipocyte differentiation through the control of PPAR γ levels (21). NEGR1 acts as the focal point of transcript network in relation to obesity (22). FTO mediates adipogenesis and adipocyte lipid content by regulating gene expression (23). TMEM18 and NEGR1 have both been implicated in obesity by regulating the central nervous system (CNS) (11). KCTD15 was shown to inhibit neural crest formation by regulate transcription factor AP-2 (24); however, the role of KCTD15 in adipogenesis has not been studied. Therefore, we focused on the role of KCTD15 in adipogenesis.

The gene expression level of KCTD15 decreased during 3T3-L1 differentiation.

We further studied the role of KCTD15 during adipogenesis by using 3T3-L1 pre-adipocytes in vitro differentiation system. Firstly, the standard induction protocol elaborated on in the method part was applied to facilitate 3T3-L1 cells to differentiate, and cells were collected at Day-2, Day 0, Day 2, Day5 and Day10. Sequentially, the expression of KCTD15 at different time points were spotted by PCR with specific PCR primers and the density of the PCR products were quantified with Quantity One software. As is shown in Fig.2A, KCTD15 expression was high at the beginning of differentiation. Meanwhile, the KCTD15 level throughout the differentiation process was determined and normalized to β -actin (Fig.2B). The results show that KCTD15 expression increased at day-0 before decreasing and reaching the lowest level in mature adipocytes (day10) (Fig.2C, 2D).

KCTD15 deficiency compromised adipocyte differentiation and lipid accumulation in 3T3-L1 cells

For further study on how KCTD15 functions in adipogenesis, the knockdown strategy was applied. Firstly, KCTD15 specific siRNA was transiently transfected into 3T3-L1 pre-adipocytes, while non-targeting siRNA was used as negative controls. As shown in Fig.3A, after siRNA transfection the expression of KCTD15 decreased obviously, suggesting the knock down efficiency for KCTD15 was high (Fig.3A). Then the cells were induced to differentiation and ten days after the induction, the extent of adipocyte differentiation was checked by Oil Red O dyeing. Intriguingly, compared to the negative control, the differentiation of KCTD deficient cell were significantly inhibited (Fig.3B). In addition, another indicator of adipogenesis, Lipid accumulation, was also measured in both the control and KCTD15 knockdown cells through measuring the triglyceride level in the cells. Consistent with the impact made by KCTD15 knockdown on adipocyte differentiation, the level of accumulated triglycerides was also lower in the KCTD15 deficient cells than the control cells (Fig.3C). These measurements were taken in triplicate and had a *P* value of 0.0220. Taken together, these data suggest that KCTD15 performs a crucial part for adipogenesis.

Discussion

Studies on the association between the candidate obesity-related genes identified by GWAS and obesity suggest the need of further research into the biological function of these genes. This promoted us to check how the genes are expressed in both the normal weight and obese subjects. So as far as the existing research is concerned, the focus of attention is the changes to mRNA levels among these candidate obesity-related genes in adipose tissue from normal weight and obese subjects. No significant changes of MTCH2, GNPDA2 and MC4R were detected. Interestingly, the expression of MTCH2, which is abundant in human WAT, was shown to be higher in obese women (25). Meanwhile, it was found that TMEM18 and FTO expression were lower in obese subjects. The expression of SH2B1 and KCTD15 were significantly decreased in obese subjects, whereas NEGR1 was increased in this group. Interestingly, it was also illustrated that the expression of KCTD15 was down-regulated in the adipose tissue of obese rats (10). We are the first to confirm that the expression of KCTD15 is less obvious among obese people but with a notable negative association with BMI displayed, which is used to identify the degree of obesity. Some studies have suggested that KCTD15 is capable of regulation of various biological processes in relation to obesity, such as glucose metabolism and fat metabolism (5, 19). However, the difference made by KCTD15 in adipogenesis is still unknown. Throughout this research, the function of KCTD15 in 3T3-L1 differentiation was investigated.

Aberrant and excessive fat accumulation is defined as obesity. Fat accumulation is determined by adipocyte number (adipocyte differentiation) and size (fat storage). Similarly, 3T3-L1 pre-adipocyte differentiation is composed of the growth inhibition of proliferating pre-adipocytes (Day 0), coordinated reentry into the cell cycle with clonal expansion (Days 1–2), postmitotic growth inhibition (Days 3–4), associated terminal differentiation and lipid accumulation (Days 4–10) (23). Among these differentiation stages, the clonal expansion stage plays a necessary and indispensable role in differentiation. With this research, it's revealed that KCTD15 was expressed at a high level during initial stages (pre-adipocytes,

Days 0–2) and then became lower in the later stages (mature adipocytes, Days 5–10). The data suggested that KCTD15 may be essential for the early mitotic events. Indeed, we demonstrated that KCTD15 knockdown in 3T3-L1 caused adipocyte differentiation and lipid accumulation to be inhibited.

Interestingly, our in vitro adipocyte differentiation study showed that KCTD15 was very important for adipocyte differentiation initiation, but, in comparison, there was a downgrade in the regulation of how KCTD15 was expressed in adipose tissues obtained from overweight human subjects when compared to those obtained from normal-weight controls. This seemingly paradoxical data may be due to the fact that white adipose tissue in human is terminally differentiated. Therefore, it is reasonable to observe the lower expression of KCTD15 in adipose tissue from overweight subjects, because our in vitro experiments clearly demonstrated that KCTD15 expression was down-regulated in the terminal stage of adipocyte differentiation.

Notably, the sequential biphasic changes in KCTD15 gene expression during adipogenesis are not unique. During adipocyte differentiation, the protein level of KCTD15 increased in the early stage and then markedly decreased in the later stage. It was also shown that knockdown of KCTD15 blocked adipocyte differentiation. It has been verified that KCTD15 can regulate how transcription factor AP-2 (24) is expressed. During adipocyte differentiation, transcription of genes that generate the adipocyte phenotype can be made active by CCAAT enhancer-binding protein- α (C/EBP α) coordinately (25–26). Before mitotic clonal expansion occurs, C/EBP α gene ends up being silenced because of the repression of AP-2 (24). We hypothesize that KCTD15 inhibit transcription factor AP-2 expression and eventually affect adipocyte differentiation via suppression of C/EBP α . However, more evidence is required to determine the underlying mechanism whereby KCTD15 regulates adipocyte differentiation.

In summary, we elucidated the expression patterns of seven obesity-associated genes in adipose tissue and obese subjects. It is discovered that KCTD15 expression level was closely related to BMI. In addition, our in vitro adipocyte differentiation study demonstrated that KCTD15 has a potential to perform a crucial part at the initial stages of adipocyte differentiation. Nevertheless, in-depth researches are required to figure out the mechanism contributing to adipocyte differentiation by KCTD15.

Conclusion

Our study suggested that KCTD15 can regulate the adipogenesis and the expression of KCTD15 is associated with obesity in humans. Therefore, KCTD15 plays a critical part in the regulation of adipogenesis and obesity.

Declarations

Ethics approval and consent to participate

The clinical trial registration number is ChiCTR-RNRC-11001441, and we were granted permission from the adult subjects.

Consent for publication

All authors agree to publish.

Availability of data and materials

The data that support the findings of this study are available

Competing interests

The authors have no conflicts of interest to disclose.

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Author's contributions

JX was responsible for the collection and preservation of clinical specimen, conduct of all the experiments and data collection and analysis; XZ was responsible for RNA and protein manipulation, adipocyte differentiation experiments; YY assist adipocyte differentiation experiments; MW was responsible for communication with patients and collection of clinical specimens as required in patient's informed consent; KBZ was responsible for analyzing statistics and modifying manuscript. ZHX was responsible for subjects design and statistical analysis and modification of manuscript and online submission.

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Figures

Figure 1

Figure 1

The mRNA expressions of eight obesity-related genes in the adipose tissue from normal and obese subjects and correlation with obesity.(A) Total RNAs from adipose tissues of 16 normal subjects and 23 obese subjects were isolated for mRNA expression measurement. Representative PCR results of the eight genes were presented. GAPDH was used as a control. (B) All PCR products were quantified by Quantity One software and normalized to GAPDH levels. The relative expressions of eight genes in all subjects

were presented as closed or open geometric figures. The values in the figure are shown as mean $\pm$ SEM. $P < 0.05$ was considered statistically significant. (C) The correlation coefficient between expressions of genes and BMI was analyzed with SPSS 16.0 software. * and ** presents Pearson correlation < 0.05 and < 0.01 respectively, indicating that the values are significantly correlated. The person value less than 0 means negative correlation while more than 0 means positive correlation. A Pearson correlation coefficient close to + 1 or - 1 indicates that the two variables are highly correlated (positively or negatively, respectively). A correlation coefficient between 0 and + 0.30 or between 0 and - 0.30 was considered of having no correlation between the two variables. (D) The expression of KCTD15 was negatively-correlated with BMI. P and r values are given for each simple regression plot.

Figure 2

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Figure 2

The expression of KCTD15 was decreased during adipocyte differentiation. (A) Differentiation of 3T3-L1 preadipocytes (Day-2) were induced by hormone cocktail. Total RNAs were isolated for quantification of KCTD15 by RT-PCR at different time points (Day-2/0/2/5/10). β -actin was used as an internal control. (B) PCR products were quantified by Quantity One software. The values were presented as density of PCR products normalized to β -actin levels. (C) Cell lysates were collected at different stages of differentiation

(Day-2/0/2/5/10) with RIPA buffer, and subjected to western blot detection. KCTD15 protein was detected with specific antibody. β -tubulin was used as a loading control. (D) Group data showing the protein expressions of KCTD15 normalized to β -tubuli expression. * $P < 0.05$ versus the stage of differentiation Day-2, # $P < 0.05$, ## $P < 0.01$ versus the stage of differentiation Day-0.

Figure 3

Figure 3

Deficiency of KCTD15 inhibits adipocyte differentiation and lipid accumulation. (A) Preadipocyte 3T3-L1 cells were transfected with negative control or KCTD15 specific siRNA, respectively, and subjected to induction of differentiation. Knockdown efficiency was tested by semi-quantity RT-PCR after 48 h. β -actin was used as an internal control. (B) Oil Red O staining of cells was performed at Day-0 and Day10 in both negative control and KCTD15 specific siRNA treated cells. (C) The triglyceride content was measured with a triglyceride kit as mg triglycerides/mg protein. Relative content represents the percentage of the knockdown group normalized by the control group. Data are presented as means $\pm$ SEM of 3 independent experiments performed in triplicate. * Statistical significance ($P < 0.05$).

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