Comparative proteome analysis reveals VPS28 regulates milk fat synthesis through ubiquitylation in bovine mammary epithelial cells

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In our previous study, we found VPS28 (vacuolar protein sorting 28 homolog) could alter ubiquitylation level to regulate milk fat synthesis in BMECs (bovine primary mammary epithelial cells). While the information on the regulation of VPS28 on proteome of milk fat synthesis is less, we explored its effect on milk fat synthesis using iTRAQ (isobaric tags for relative and absolute quantitation) assay after knocking down VPS28 in BMECs. We found 2,773 proteins in three biological replicates with less than in 1.2% false discovery rate were identified and quantified. Among them, a subset of 203 proteins were screened as significantly down- (111) and up- (92) regulated inVPS28 knockdown BMECs compared with the control groups. According to Gene Ontology analysis, the differentially expressed proteins were enriched in the "proteasome", "ubiquitylation", "metabolism of fatty acids", "phosphorylation", and "ribosome". Meanwhile, some changes occurred in the morphology of BMECs and an accumulation of TG (triglyceride) and dysfunction of proteasome were identified, and a series of genes associated with milk fat synthesis, ubiquitylation and proteasome pathways were analyzed by RT-QPCR (quantitative real-time PCR). The results of this study suggested VPS28 regulated milk fat synthesis was mediated by ubiquitylation, it could be an important new area of study for milk fat synthesis and other milk fat content traits in bovine.

1 **Comparative proteome analysis reveals VPS28 regulates milk fat**

2 **synthesis through ubiquitylation in bovine mammary epithelial cells**

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Abstract

In our previous study, we found VPS28 (vacuolar protein sorting 28 homolog) could alter

ubiquitylation level to regulate milk fat synthesis in BMECs (bovine primary mammary

epithelial cells). While the information on the regulation of VPS28 on proteome of milk fat

synthesis is less, we explored its effect on milk fat synthesis using iTRAQ (isobaric tags for

relative and absolute quantitation) assay after knocking down VPS28 in BMECs. At a false

discovery rate of 1.2%, We found 2,773 proteins in three biological replicates. Among them, a

subset of 203 proteins were screened as significantly down- (111) and up- (92) regulated

inVPS28 knockdown BMECs compared with the control groups. According to Gene Ontology

analysis, the differentially expressed proteins were enriched in the "proteasome",

"ubiquitylation", "metabolism of fatty acids", "phosphorylation", and "ribosome". Meanwhile,

some changes occurred in the morphology of BMECs and an accumulation of TG (triglyceride)

and dysfunction of proteasome were identified, and a series of genes associated with milk fat

synthesis, ubiquitylation and proteasome pathways were analyzed by qRT-PCR (quantitative

real-time PCR). The results of this study suggested VPS28 regulated milk fat synthesis was

mediated by ubiquitylation, it could be an important new area of study for milk fat synthesis and

other milk fat content traits in bovine.

Keywords: VPS28; milk fat synthesis; iTRAQ; proteome; ubiquitylation

Introduction

VPS28 is a member of the class E VPS proteins, and also is a major component of ESCRT I

(endosomal sorting complexes required for transport I). ESCRT-0, ESCRT-I, ESCRT-II,

ESCRT-III and some auxiliary components constitute ESCRTs, play crucial roles in

concentration and sorting of ubiquitinated proteins of the MVB (multivesicular body) for

incorporation into ILV (intralumenal vesicles) (*1-3*). The importance of ESCRTs was

demonstrated by Raymond *et al.*, who showed that disruption of ESCRTs resulted in an

accumulation of membrane proteins and no longer degraded in the vacule (*4*). Recent studies

showed that ESCRTs play a critical role in degradation of ubiquitinated proteins through

lysosome and proteasom (*5, 6*). Particularly, VPS28 is localized to ubiquitin-rich endosomes

during ligand-induced receptors internalization and contributes directly to receptor trafficking (*7*).

In previous studies, we found a SNP in the 5' UTR region of *VPS28* showed a very strong

association with milk fat percentage and its expression level significantly affected milk fat

synthesis in Chinese Holstein (*8-11*). Based on the structural feature and function of ESCRTs,

we believed that VPS28 could regulate milk fat synthesis through engaging ESCRTs complexes

to affect the ubiquitin-mediated degradation of proteins, which has been proved in our previous

study (*8, 12*). However, the molecular mechanisms of VPS28 response to milk fat synthesis still

remain unclear. Thus, in present study, to better understand the mechanisms, we used iTRAQ

technology that allows quantitative comparisons of protein abundance to much greater insight

into the regulation of VPS28 on milk fat synthesis in Chinese Holstein. After performing RNAi

61 experiment in BMECs, we compared the knockdown BMECs groups with the control groups to

62 identify differentially expressed proteins by iTRAQ. Changes in the expression patterns of the

63 proteins could provide a basis for clarifying the molecular mechanisms for VPS28 regulating

64 milk fat synthesis in Chinese Holstein, which may be a forward step for milk fat synthesis

65 regulation, In addition, this study can provide a reference for elucidating the molecular

66 mechanisms of milk fat traits.

Materials and Methods

67

Animals

68

69 The procedures of collecting BMECs from the mammary tissues of Chinese Holstein cows

70 according to the Animal Welfare Committee of Shandong Agricultural University (Permit

71 Number is SDAUA-2018-022).

Cell Culture

72

73 Cell culture experiments were performed using primary BMECs. Chemicals were purchased

74 from Life Technologies (Carlsbad, CA, USA) unless noted otherwise. Primary BMECs were

75 kept in our laboratory. BMECs were plated in serum-containing medium DMEM-F12

76 supplemented with 10 kU/mL penicillin, 10mg/mL streptomycin, 10% fetal bovine serum (FBS)

77 and 1% ITS -G (1mg/mL Insulin, 0.55mg/mL Transferrin, 0.67mg/L Selenium Solution). All

78 cells were cultured on plastic cell culture plates at 37℃ in a humidified atmosphere containing

79 5% CO_2.

Knockdown of VPS28 via RNAi in BMECs

80

81   Stealth RNAi™ siRNAs targeting the bovine VPS28 gene open reading frame were

82   designed and synthesized by GenePharma Corporation (Shanghai, China). One day prior to

83   transfection, BMECs were seeded without antibiotics. When cells reached 80% confluence,

84   VPS28-siRNAs (GUCCAGGGCUCAGAAAUCATT and GACGUGGUCUCGCUCUUUATT)

85   as tandem constructs, were transfected in to BMECs using X-treme GENE siRNA Transfection

86   Reagent (Roche, Penzberg, Germany) at a 1:10 molar ratio. Cells were harvested at 72 hours

87   after transfection for mRNA analysis via real-time quantitative PCR (RT-qPCR).

88   ***Sample Preparation***

89   Six BMECs samples from two groups (control and VPS28 knockdown) were incubated in

90   lysis buffer (7 M urea, 2 M thiourea and 0.1% CHAPS) for 30 min on ice and sonicated (80W,

91   ultrasonic 0.2s, intermittent 2s, a total 60s) on ice. Cells debris was pelleted by centrifugation at

92   15,000 g for 20 min at 4℃. The supernatants were collected and stored at -80 ℃. The protein

93   concentration was determined using Bradford assay (Sigma-Aldrich, USA).

94   ***Protein digestion and iTRAQ labeling***

95   Protein digestion was performed using the filter aided sample preparation (FASP) method.

96   Each protein extract (200 µg) was mixed with 4 µL reducing reagent (AB Sciex, USA) for 1

97   hour at 60 ℃ and 2 µL cysteine-blocking reagent for 10 min at room temperature, the alkylated

98   protein solution was added to 10 K ultrafiltration tube and discarded the filtrate after centrifuging

99   at 12,000 g for 20 min. Then 100 µL dissolution buffers were added to the filtered unit and the

100   solution was centrifuged again at 12,000 g for 20 min and repeated 3 times. After incubating

101   overnight, the units were transferred to new collection tubes, and then adding 4 µg trypsin

102   (protein to enzyme ratio 50: 1 w/w) and mixed them at 37 ℃ for overnight. The units were

103   centrifuged at 12,000 g for 20 min discarded the filtrate, then added 50 µL dissolution buffer 5

104   and centrifuged 12,000 g for 20 min incubated at room temperature. Finally, the extracted

105   peptides were collected from bottom.

106   The iTRAQ labeling was performed according to the manufacturer's protocol (AB, Sciex).

107   After trypsin digestion, the peptides were transferred to vials containing individual iTRAQ

108   regents by incubation at room temperature for 2 hours, which was thawed and reconstituted in

109   150 µL isopropanol per one unit. The three knock-down VPS28 groups were labeled with

110   iTRAQ 115, 116 and 117; the three WT groups were labeled with iTRAQ 118, 119 and 121,

111   respectively.

112   ***Peptide fractionation with SCX (strong cation exchange) chromatography***

113   The iTRAQ labeled peptides were fractionated by SCX using RIGOL L-3000 HPLC system

114   (RIGOL, Beijing, China). The dried peptide was dissolved with 100 µL buffer A (98% ddH$_2$O,

115   2% acetonitrile) and the solution was centrifuged at 14,000 g for 20 min, the supernatants were

116   collected. The peptides were eluted at a flow rate of 0.7 ml/min with a buffer B (98% acetonitrile,

117   2% H$_2$O) gradient of 5% at 0-5 min, 8% at 5-35 min, 18% at 35-62 min, 32% at 62-64 min, 95%

118   at 64-68 min, 5% at 72 min. The elution was monitored by absorbance at 214 nm.

119   ***Quantitative analysis of proteins by iTRAQ LC-MS/MS***

120   Each collected component of the processed SCX fractions was redissolved with 20 µL 2%

121   methanol and 0.1% formic acid, and the solution was centrifuged at 12,000 g for 10 min, the

122   supernatants were collected. 10 µL solution was trapped on a precolumn (100 µm x 2 cm) and

123 then eluted on an analytical column (75 μm x 12 cm) for separation. The precolumn was packed

124 with Acclaim PepMap-C18 5 μm and analytical column was packed with EASY-Spray-C18 3

125 μm. The peptides were separated over 90 min and eluted at a flow rate of 350 nl/min. The MS

126 analysis was performed using an Applied Biosystems Q-Exactive mass spectrometer.

127   The BMECs iTRAQ identification and quantification analysis were obtained using

128 Proteome Discoverer1.3 (Thermo, USA). Proteome Discoverer1.3 was set up to search the NCBI

129 *Bos taurus* major database assuming the digestion enzyme trypsin. The differential expressed

130 proteins were accepted if they have been identified with greater than 95% confidence in all

131 iTRAQ preparations, and have ≥ 1.2 or ≤ 0.83 fold changes (iTRAQ ratios (VPS28 knockdown)-

132 115+116+117: (control)-118+119+121)in addition to $P \leq 0.05$. Gene ontology (GO) was used

133 to annotate the proteins under the biological progress (BP), molecular function (MF) and cellular

134 components (CC) GO categories (DAVID, https://david.ncifcrf.gov/) in the BMECs.

135 ***Microscopy analysis***

136   The control and VPS28 knockdown BMECs were collected and fixed with 2.5%

137 glutaraldehyde at 4℃for overnight, and washed by PBS (pH 7.0, 0.1 M) for three times. And

138 then the BMECs was fixed with 1% osmium tetroxide for 1-2 h, washed by sodium cacodylate

139 buffer, and then dehydrated with gradient alcohol until complete, finally embedded in Epon 812.

140 The fixed BMECs were cut into 1-um-thick sections and stained with uranyl acetate and lead

141 citrate.The ultrathin sections were examined under JEM-1400 electron microscope (JEOL,

142 Japan).

143 ***Measurement of cellular TG content and proteasome activities***

144   The control and VPS28 knockdown BMECs were collected and broken by ultrasonication.

145   The total lipids were extracted using the TG assay Kit (Nanjing Jiancheng Bioengineering

146   Institute, China) and monitored with Infinite M200 Reader (Tecan, Switzerland).according to the

147   manufacturer's instructions.

148   The proteasome activities (Chymotrypsin-Like, Caspase-Like and Trypsin-Like) were

149   measured using the Proteasome-Glo™ Cell-Based Assays (Promega, Mannheim, Germany)

150   according to the manufacturer's instructions, and the fluorescence intensity was monitored with

151   Infinite M200 Reader (Tecan, Switzerland).

152   ***Real-time quantitative PCR analysis***

153   The primers of selected genes for RT-qPCR were designed with Primer 5.0 and synthesized

154   by The BGI (Beijing Genomics Institute) Co., Ltd. The GAPDH (glyceraldehyde-3-phosphate

155   dehydrogenase) gene was used as the control. The primer sequences are listed in Table 1. RNA

156   extraction, cDNA synthesis and RT-qPCR were performed according to the manufacturer's

157   instructions, and were repeated three times. The relative expression of genes was computed using

158   the $2^{-\Delta\Delta Ct}$ method

159   ***Statistical Analysis***

160   R-package (R v3.02) was conducted to evaluated changes between VPS28 knockdown

161   BMECs groups and the control groups. And differences were declared significant at $P \leqslant 0.05$.

Results

163   ***VPS28 knockdown alters expression of multiple proteins in BMEC***

164   VPS28 expression in BMEC were down-regulated by 78% with tandem constructs (as

165   shown in Fig. 1A), and then, to obtain a whole picture of the proteomic changes in VPS28

166   knockdown BMEC, we conducted iTRAQ experiment in combination with LC-ESI-MS/MS

167   analysis to investigate differentially expressed proteins in VPS28 knockdown BMECs groups

168   (labeled iTRAQ-115, 116 and 117) and the control groups (labeled iTRAQ-118, 119 and 121).

169   At a false discovery rate of 1.2%, a total of 2,773 proteins were identified from 14,031 peptides.

170   The peptides of all proteins are provided in Supporting Information Table S1.

171   To further understand the differentially expressed proteins after knocking down VPS28 in

172   BMECs and basing on standard of the differentially expressed proteins, a total of 203 distinct

173   proteins were identified by iTRAQ analysis in VPS28 knockdown BMECs (Detailed gene

174   information and fold-change following VPS28 knockdown were provided in Table 2). 92

175   proteins were significantly up-regulated ($\geq$1.2-fold) while 111 proteins were significantly

176   down-regulated ($\leq$0.83-fold) when compared with the control BMECs.

177   The DEPs were categorized into 53 clusters ($P < 0.05$, as shown in Table 3) according to

178   their biological processes (BPs), cellular components (CCs) and molecular functions (MFs). The

179   top 6 GO terms for BPs were enriched in cytoplasmic translation (GO: 0002181), translation

180   (GO: 0006412), cholesterol homeostasis (GO: 0042632), cholesterol efflux (GO: 0033344),

181   positive regulation of cholesterol esterification (GO: 0010873) and high-density lipoprotein

182   particle assembly (GO:0034380). These biological processes were involved in the lipid

183   metabolism and transportation. The top 5 GO terms for CCs were cytosolic large ribosomal

184   subunit (GO: 0022625), extracellular exosome (GO: 0070062), focal adhesion (GO: 005925),

185 membrane (GO: 0016020) and very-low-density lipoprotein particle (GO: 0034361). These

186 cellular components were response to the ubiquitin system. The top 5 GO terms for MFs were

187 mainly enriched in structural constituent of ribosome (GO: 0003735), RNA binding (GO:

188 0003723), cholesterol transporter activity (GO: 0017127), phosphatidylcholine-sterol O-

189 acyltransferase activator activity (GO: 0019843). These results showed that the DEPs following

190 VPS28 knockdown were mainly involved in the functions of transport and metabolism of lipid,

191 lipoprotein and lipoprotein receptor binding, and ribosome translation.

192 ***Effect of VPS28 knockdown on morphology of BMECs***

193 Electron micrographs could observe the morphological changes in BMECs. Compared with

194 the control BMECs groups, the VPS28 knockdown groups showed containing more and

195 strikingly large lipid droplets and many luminal spaces were completely filled with aggregated

196 lipid (as shown in Fig. 1 B and C). And in parallel, the content of TG was increased by 3.3-fold

197 above the control BMECs groups (Fig.1D).

198 The GO analysis demonstrated DEPs enriched in ubiquitylation singaling, and

199 ubiquitylation mediates the degradation of membrane proteins and intracellular proteins, which

200 plays an crucial role in receptor-mediated signaling pathways and quality control of intracellular

201 proteins. And then we examined the proteasome activity (chymotreypsin-like activity, caspase-

202 like activity, trypsin-like activity) after knocking down VPS28 (as shown in Fig. 2). The results

203 showed that VPS28 knockdown could significantly decrease the three activites of proteasome,

204 the relative activities of chymotreypsin-like, caspase-like and trypsin-like are 0.60, 0.64, 0.74,

205 respectively. And we also found the level of ubiquitinated proteins was increased by VPS28

206 knockdown (the data has published) (*12*). These results indicated that VPS28 could regulate

207 ubiquitylation-proteasome system.

208 ***Validation of gene expression by RT-qPCR***

209 To investigate whether the alteration of proteins expression level were the result of

210 transcriptional regulation, we detected mRNA levels of five selected proteins and five genes that

211 were related to metabolism of fatty acids, ubiquitylation and proteasome pathways. The RT-

212 qPCR results showed high qualitative and quantitative concordance (correlation coefficient

213 >0.95). As shown in Fig.3, *CD36* (cluster of differentiation 36) in fatty acids taken up process,

214 *FASN* (fatty acid synthase), *SCD* (stearoyl-CoA desaturas) and *DGAT1* (diacylglycerol

215 acyltransferase 1) in fatty acids synthesis pathway, *ADFP* (adipose differentiation-related protein)

216 in lipid droplet secretion process, were all up-regulated by VPS28 knockdown. *ACACA* (acetyl-

217 CoA) in *de novo* fatty acids synthesis pathway was down-regulated in VPS28 knockdown

218 BMECs. *PSMG1* (proteasome assembly chaperone 1) in proteasome system, *RPS29* (ribosomal

219 protein S29) in ribosome translation pathway, *UBE2L* (ubiquitin-conjugating enzyme E2L),

220 *ISG15* (interferon-stimulatory gene ISG15) in ubiquitylation pathway, were all up-regulated by

221 VPS28 knockdown. The results showed that the mRNA expression levels of genes were

222 generally corresponded with the changes in the morphology of BMECs and proteins expression

223 detected by the iTRAQ approach.

224 **Discussion**

225 Milk fat synthesis is a complex process. Numerous types of molecular and chemical

226   relationships exist which directly or indirectly could affect protein activity and regulate milk fat

227   synthesis, such as ubiquitylation and protein-protein interaction. Ubiquitylation is an important

228   post-translational modification and it can mediate the intercellular proteins degradation which

229   plays a crucial role in receptor-mediated signaling pathways. VPS28 as a subunit of ESCRTs is

230   crucial for ubiquitin-mediated degradation of proteins, and we found VPS28 could alter the

231   ubiquitylation level to regulate milk fat synthesis in previous studies (*8, 12*). However, much less

232   is understood regarding the molecular mechanisms of VPS28 regulating milk fat synthesis

233   through ubiquitylation. In this study, iTRAQ technology were performed to accurately identify

234   the peptides and precisely quantify the iTRAQ labels. Subsequently, cluster and pathways

235   analysis were devoted to obtain consistent results to further elucidate the regulation pathways of

236   VPS28 on the milk fat synthesis.

237   The ubiquitin system is a protein degradation pathway, dedicates to the ubiquitylation of

238   cellular targets and the subsequent control of numerous cellular functions and plays an important

239   role in metabolism regulation(*13*). The deregulation of components of this elaborate network

240   leads to an accumulation of membrane proteins and no longer degraded in the vacule. Numerous

241   studies indicated that, as one subunit of ESCRTs, VPS28 played a crucial role in ubiquitin-

242   mediated degradation of membrane proteins (*5, 6*) and cytoplasmic proteins (*14*). In this study,

243   BMECs sections showed that the form of fat droplets was affected after knocking down VPS28,

244   and we found an accumulation of ubiquitinated proteins and a dysfunction of proteasome activity

245   in VPS28 knockdown BMECs groups. The proteomic analysis indentified many differentially

246   expressed proteins that were considerably enriched in extracellular exosome (GO: 0070062) and

247   membrane (GO: 0016020). These GO categories were associated with the ubiquitylation system.

248   These indicated that VPS28 knockdown played a crucial role in ubiquitylation.

249   In BMECs, fatty acids taken up and *de novo* fatty acids synthesis are involved in milk fat

250   synthesis. In our previous study, by knocking down *VPS28* in BMECs, we found ubiquitinated

251   CD36 level was increased significantly which is the main protein involved in long-chain fatty

252   acids uptake, and the mRNA expression of other milk fat-related genes were also up-regulated.

253   These results indicated the process of long-chain fatty acids taken up was promoted by VPS28

254   knockdown in BMECs. In parallel, the expression of ADFP was found increased in VPS28

255   knockdown BEMCs. ADFP as a specific marker for lipid droplet, its expression level is in

256   keeping with with abundance of lipid droplets in cell (*15*). The proteomic analysis also

257   indentified many differentially expressed proteins enriched in lipid metabolism. These data

258   confirmed VPS28 knockdown could facilitate milk fat synthesis in BMECs.

259   The DEPs analysis indicates that VPS28 could regulate milk fat synthesis in two approaches,

260   the one is VPS28 directly regulates milk fat synthesis through ubiquitylation and the other one is

261   VPS28 mediates ubiquitin-proteasome system to regulate milk fat synthesis. To further

262   understand these, we used the key interact proteins and genes to generate the pathway networks,

263   following is the description of the model presented in Fig. 4:

264   **VPS28 knockdown leads an accumulation of ubiquitinated membrane proteins to**

265   **promote fatty acids taken up to synthesize TG:** In this regulation, CD36 appears to be the

266   most important protein, and the other enzymes involved in milk fat synthesis could be increased

267   through allosteric effect. CD36 as a membrane scavenger receptor was identified as a receptor of

268 fatty acid and ubiquitinated CD36 facilitates long-chain fatty acids uptake (*16-18*). Following

269 VPS28 knockdown, the accumulation of ubiquitinated CD36 could import more long-chain fatty

270 acids into BMECs, and the imported long-chain fatty acids are combined and transported to

271 endoplasmic reticulum by FABP (fatty acid binding proteins). Subsequently, SCD and DGAT1

272 are induced to utilize fatty acids to synthesize TG. Therefore, VPS28 knockdown could promote

273 long chain fatty acids taken up to synthesize TG.

274 **VPS28 knockdown leads an accumulation of ubiquitinated cytoplasmic proteins to**

275 **promote de novo biogenesis, activation and channelling of fatty acids to synthesize TG.** In

276 this regulation, proteasome plays the most important role. Following VPS28 knockdown,

277 proteasome activity and the expression of ISG15 (interferon-stimulatory gene ISG15) were

278 decreased. ISG15 is an ubiquitin-like protein that mediates the conjugation of different proteins

279 through its ISGylation enzymes UBE2L6 (ubiquitin conjugating enzyme E2 L6) (*19*), and we

280 also found UBE2L6 was down regulated. Previous studies have suggested that down-regulation

281 of ISG15 and UBE2L6 can counteract degradation of triglyceride lipase (*20-22*), and ISG15

282 conjugation regulates exosome secretion(*23*). The accumulation of ACACA (Acetyl-CoA) (*24*)

283 and the other allosteric affected enzymes promote the de novo biogenesis, activation and

284 channelling of fatty acids to synthesize TG in BMECs.

Conclusions

286 In this study, iTRAQ technology was used to demonstrate proteome spectrum changes in

287 the BMECs after knocking down VPS28. It was concluded that VPS28 knockdown promotes

288   milk fat synthesis in BMECs which might be attributed to differentially expressed proteins. The

289   DEPs enriched in gene ontology categories associated with ubiquitylation likely played an

290   important role in the TG synthesis in BMECs. The dysfunctional proteasome, accumulation of

291   TG might explain the regulation of VPS28 on milk fat synthesis was mediated by ubiquitylation.

292   Our results provide a comprehensive dataset of ubiquitylation regulating milk fat synthesis, and

293   also provide a reference for the further study of ubiquitination in dairy breeding.

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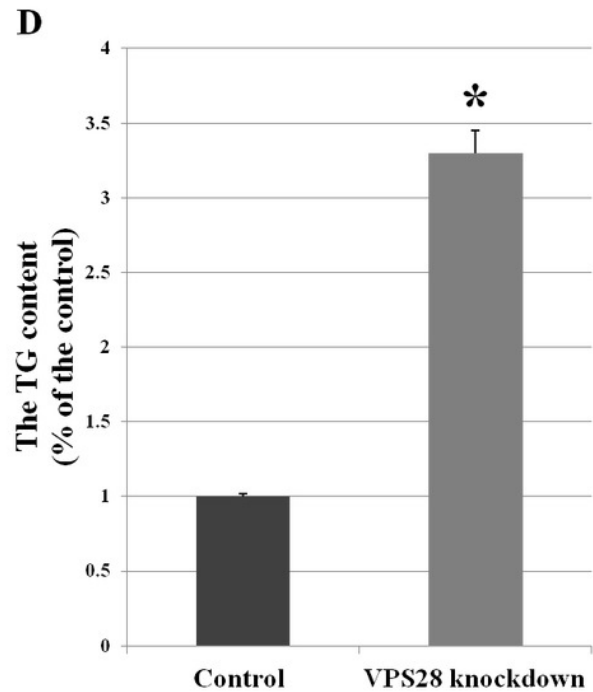
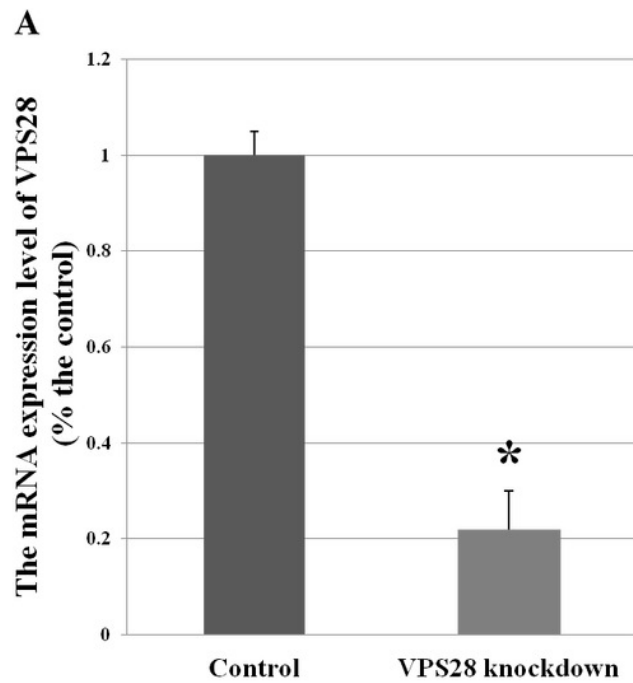
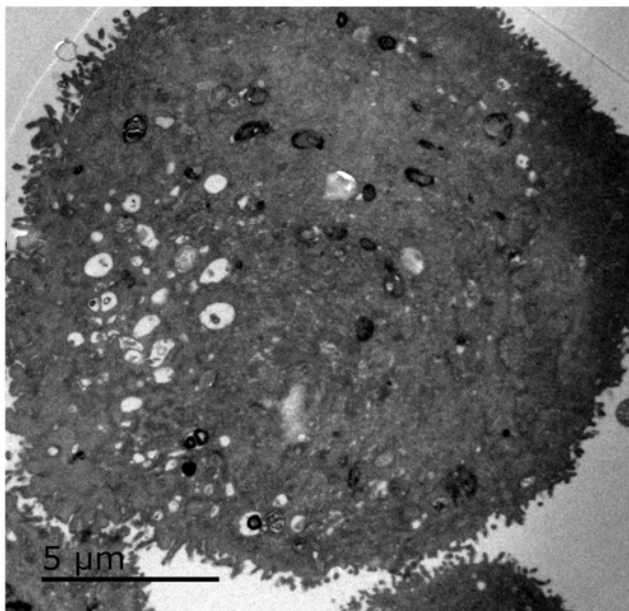
381

Figure 1

Effects of VPS28 knockdown on BMECs.

A: The mRNA expression of VPS28 was decreased by tandem siRNAs constructs. B and C: Electron micrographs of BMECs. D: The TG content was significantly increased in VPS28 knockdown BMEC. Data are averages of three replicates. The error or bars denote SEM. * indicates the difference is significant ($P \leq 0.05$).

B : Control

C: VPS28 knockdown

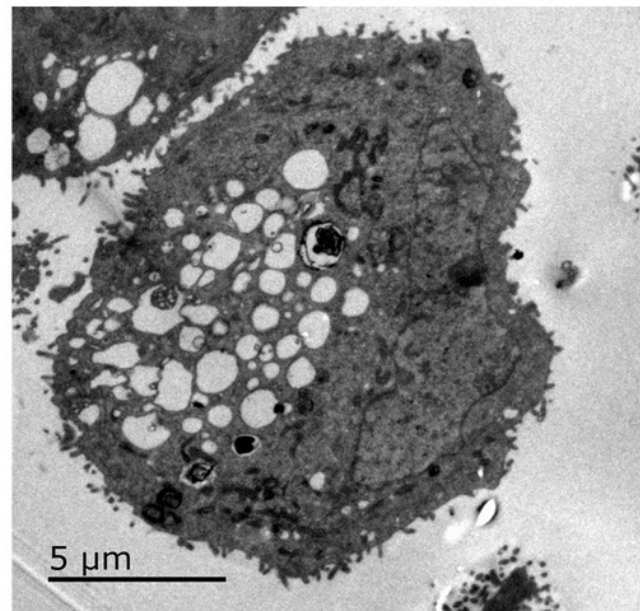



Figure 2

The proteasome activity was decreased by VPS28 knockdown.

Chymotreypsin-like activity, caspase-like activity and trypsin-like activity are the three activities in proteasome. * indicates the difference is significant ($P \leq 0.05$).

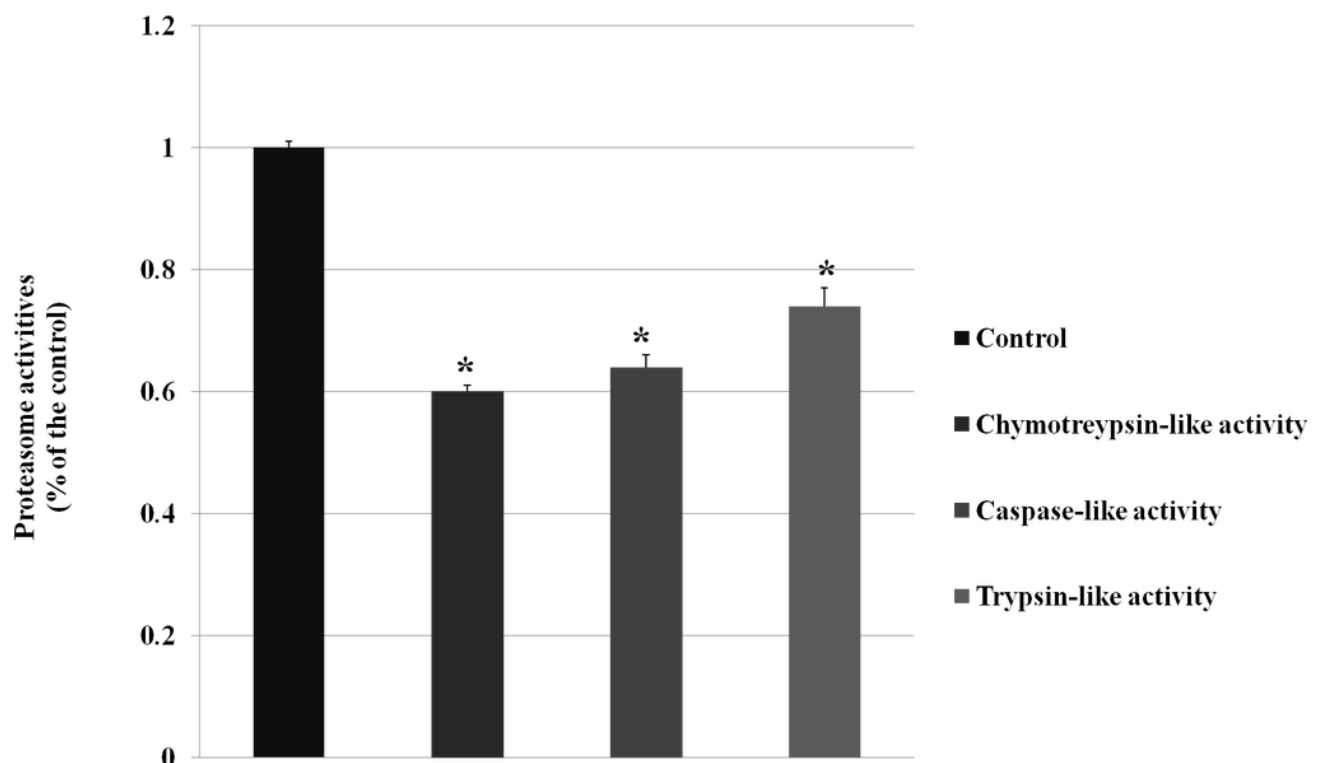



Figure 3

The mRNA expression of selected genes in VPS28 knockdown BMECs.

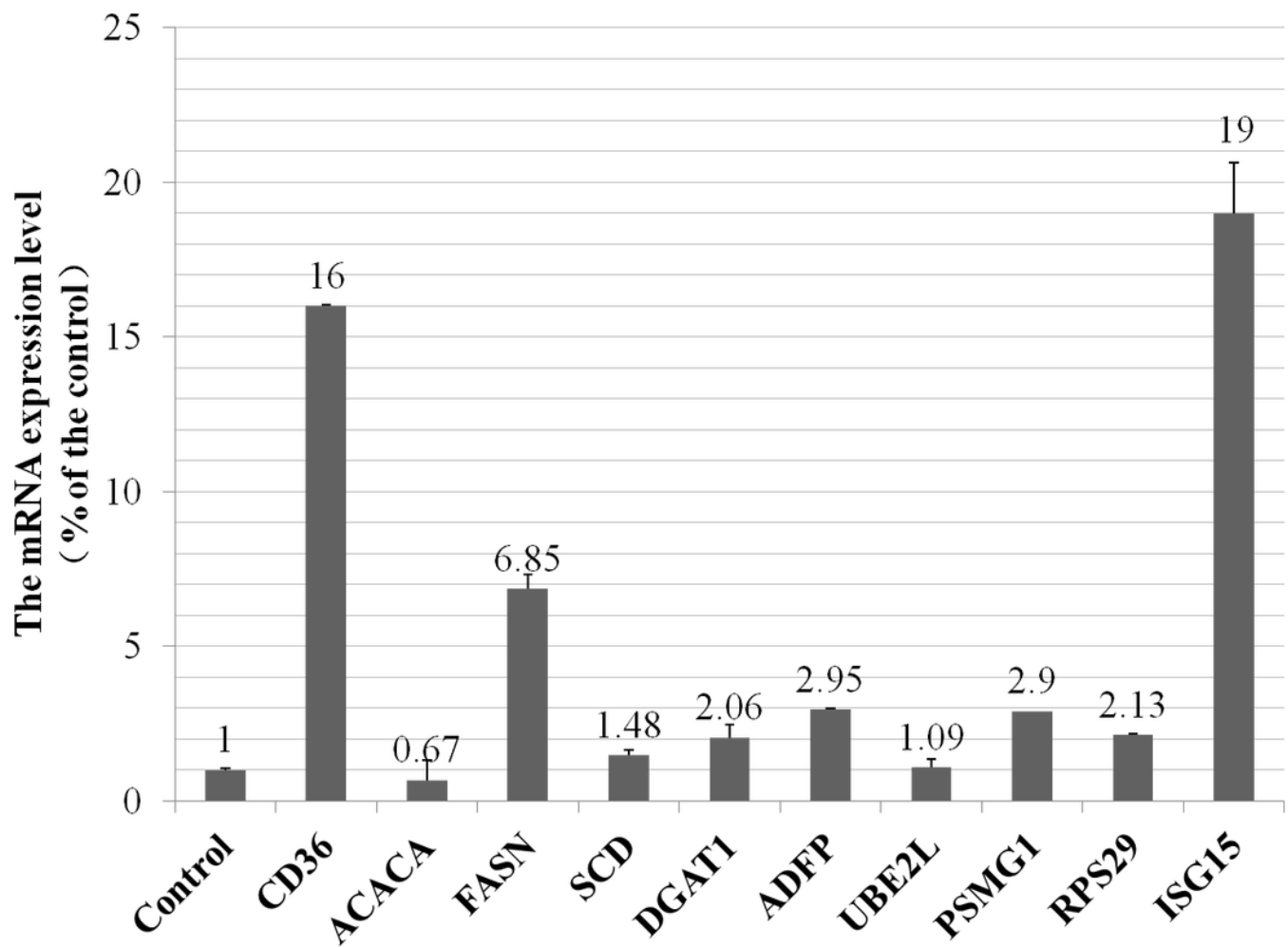
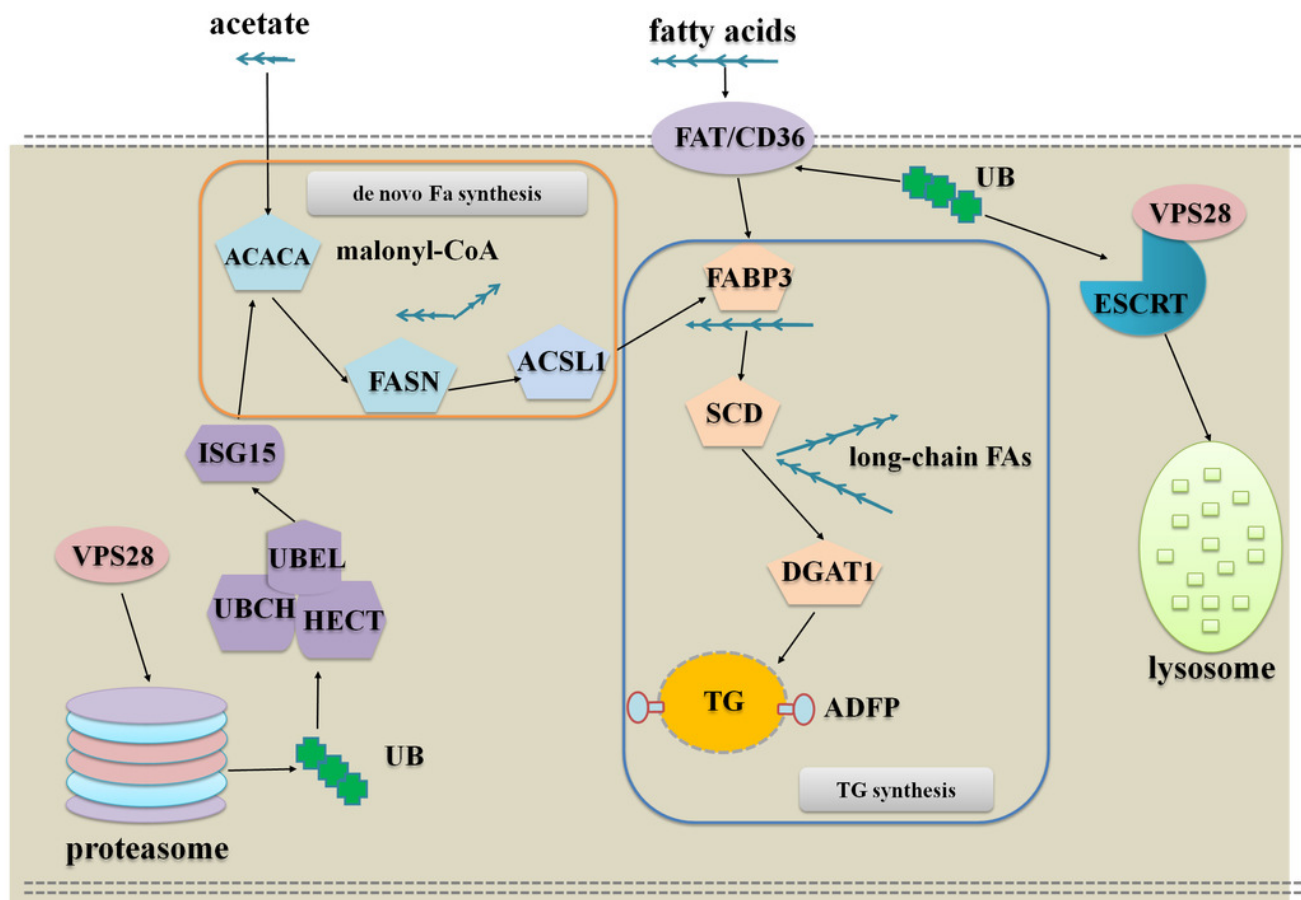



Figure 4

The network of VPS28 knockdown regulates milk fat synthesis in BMECs.

Differentially expressed proteins following VPS28 knockdown in BMEC.

1 **Table 1. Primers of the selected genes for qRT-PCR and their relative expression.**

Genes	Primer sequences(5'→3')	Relative expression
GAPDH	AGATGGTGAAGGTCGGAGTG	/
	CGTTCTCTGCCTTGACTGTG	
VPS28	GGAAACAAGCCGGAGCTGTA	0.22
	CTGGATCTCGTCCATGGCTC	
CD36	GACGGATGTACAGCGGTGAT	16.00
	GAAAAAGTGCAAGGCCACCA	
ACACA	AGTGTTCTGATCAGGTCTTCTTGT	0.67
	GGGAGGCAAAAACCTCCAGA	
FASN	AGGCGTGCGTGACACTT	6.85
	AATACAGTTGGCCGTCACCA	
SCD	TCCTGATCATTGGCAACACCA	1.48
	CCAACCCACGTGAGAGAAGAA	
DGAT1	TACCCCGACAACCTGACCTA	2.06
	GGGAAGTTGAGCTCGTAGCA	
ADFP	GCGTCTGCTGGCTGATTTC	2.95
	AGCCGAGGAGACCAGATCATA	
PSMG1	GGGAAGAAGTCGGTTGTGCT	2.87
	AAAAAGCCTCTGTGGGGGAC	
UBE2L	CTGGCACAGTATATGAAGACCTGA	1.28
	GGTAGCAGGGTGTGAGGAAC	
RPS29	TGTTTCCGCCAGTATGCGAA	2.13
	GCTGGATGAGCCATCTAAGGAA	
ISG15	CCATCCTGGTGAGGAACGAC	19.02
	GTCTGCTTGTACACGCTCCT	

3

Gene ontology analysis of differentally expressed proteins in VPS28 knockdown BMECs.

1 **Table 2. Differentially expressed proteins following VPS28 knockdown in BMEC.**

Accession	Description	RATIO
300796460	pescadillo homolog	0.79
333440457	immortalization up-regulated protein	1.38
528937065	PREDICTED: fragile X mental retardation syndrome-related protein 1 isoform X6	0.83
741896620	PREDICTED: bifunctional coenzyme A synthase isoform X2	1.25
741972182	PREDICTED: serpin B8 isoform X1	1.33
359069079	PREDICTED: apoptotic chromatin condensation inducer in the nucleus isoform X5	0.74
528978576	PREDICTED: lysosomal acid phosphatase isoform X3	0.8
77736117	actin, alpha cardiac muscle 1	1.33
741976470	PREDICTED: actin filament-associated protein 1-like 2 isoform X5	0.82
156120791	A-kinase anchor protein 8	0.62
155371939	putative N-acetylglucosamine-6-phosphate deacetylase	1.23
741911242	PREDICTED: AP-2 complex subunit sigma-like	0.82
115496866	AP-3 complex subunit beta-1	1.22
75832056	apolipoprotein A-I preproprotein	0.46
114052298	apolipoprotein A-II precursor	0.55
741944057	PREDICTED: apolipoprotein B-100 isoform X3	0.71
27806739	apolipoprotein E precursor	0.34
51491835	ovarian and testicular apolipoprotein N precursor	0.61
528973530	PREDICTED: ADP-ribosylation factor GTPase-activating protein 1 isoform X3	0.8
300798482	rho GTPase-activating protein 35	1.36
329664977	AT-rich interactive domain-containing protein 1A	0.83
529009701	PREDICTED: acid ceramidase isoform X1	1.62
329663402	ATPase family AAA domain-containing protein 1	0.76
741980112	PREDICTED: atlastin-3 isoform X1	0.83
60101829	ATP synthase subunit 8 (mitochondrion)	1.24
28603752	ATP synthase subunit e, mitochondrial	0.63
116004323	ataxin-10	0.83
41386683	beta-2-microglobulin precursor	0.82
84000125	B-cell receptor-associated protein 29	1.34
27806229	2-oxoisovalerate dehydrogenase subunit alpha, mitochondrial precursor	0.83
741929024	PREDICTED: uncharacterized protein C4orf3 homolog	1.35

	isoform X1	
741945468	PREDICTED: calcium-binding protein 39-like isoform X1	0.79
45439308	CD63 antigen	1.36
78042548	CD81 antigen	1.25
741967799	PREDICTED: LOW QUALITY PROTEIN: serine/threonine-protein kinase MRCK beta isoform X2	1.37
529002260	PREDICTED: CUB domain-containing protein 1	0.8
77735577	CCR4-NOT transcription complex subunit 7	0.77
741922497	PREDICTED: collagen alpha-3(VI) chain isoform X7	0.81
114052042	COMM domain-containing protein 1	1.23
741945876	PREDICTED: COMM domain-containing protein 6 isoform X2	0.68
528966533	PREDICTED: COP9 signalosome complex subunit 2 isoform X1	0.82
149642865	COP9 signalosome complex subunit 3	0.74
330688478	crooked neck-like protein 1	1.6
262073106	cathepsin D precursor	1.23
118151448	CUGBP Elav-like family member 2	1.25
741917150	PREDICTED: cytochrome P450 20A1 isoform X1	1.27
164420721	dynactin subunit 5	1.29
528937089	PREDICTED: DCN1-like protein 1 isoform X1	0.67
149642575	ATP-dependent RNA helicase DDX24	1.54
114051872	density-regulated protein	0.81
157427916	H/ACA ribonucleoprotein complex subunit 4	1.22
115497846	deoxyhypusine hydroxylase	1.48
528989517	PREDICTED: developmentally-regulated GTP-binding protein 1 isoform X1	1.47
114051994	dysbindin	1.35
329663806	cytoplasmic dynein 1 light intermediate chain 2	1.23
56710336	dynein light chain 1, cytoplasmic	0.83
77735949	3-beta-hydroxysteroid-Delta(8),Delta(7)-isomerase	1.24
62751595	translation initiation factor eIF-2B subunit beta	0.71
300794424	eukaryotic translation initiation factor 5	0.78
329664532	ephrin type-A receptor 2 precursor	0.74
77735625	enhancer of rudimentary homolog	0.81
27806943	coagulation factor V precursor	0.81
528957418	PREDICTED: protein FAM114A2 isoform X1	1.29
329663573	protein FAM134A	0.75

359069460	PREDICTED: protein FAM98B	0.8
29135293	farnesyl pyrophosphate synthase	0.77
77736507	mitochondrial fission 1 protein	1.27
156718120	fat storage-inducing transmembrane protein 2	1.23
27806621	ferritin heavy chain	0.8
114051796	glucosylceramidase precursor	0.81
84000253	glutamate--cysteine ligase regulatory subunit	1.21
114051291	GDP-L-fucose synthase	0.78
741919465	PREDICTED: lysosomal protein NCU-G1 isoform X2	0.81
115496402	glucosamine-6-phosphate isomerase 2	0.83
297488836	PREDICTED: histone H1x	0.82
116812902	hemoglobin subunit alpha	0.55
17985949	hemoglobin subunit beta-1 [Rattus norvegicus]	1.23
741905547	PREDICTED: host cell factor 1 isoform X9	1.26
114052627	hepatocyte growth factor-regulated tyrosine kinase substrate	1.21
134085671	histone H1.2	0.55
155371863	histone H1.3	0.54
741971316	PREDICTED: histone H2A type 1-J	1.29
157785601	histone H2B	0.82
115496175	high mobility group protein HMG-I/HMG-Y	0.8
77736489	non-histone chromosomal protein HMG-14	0.79
297477251	PREDICTED: heterogeneous nuclear ribonucleoprotein A0	0.83
375364520	HCLS1-binding protein 3	1.48
41386699	heat shock-related 70 kDa protein 2	1.23
529014943	PREDICTED: immunoglobulin-binding protein 1 isoform X2	1.23
27805955	ubiquitin-like protein ISG15	0.83
157427772	involucrin	1.25
195539527	keratin 15	1.24
77736483	ragulator complex protein LAMTOR1	0.82
741894288	PREDICTED: galectin-7	1.26
528952868	PREDICTED: LIM and calponin homology domains-containing protein 1 isoform X5	1.36
115497506	LIM and cysteine-rich domains protein 1	1.23
686713724	PREDICTED: LOW QUALITY PROTEIN: collagen alpha-4(VI) chain-like, partial [Pongo abelii]	0.72
741878073	PREDICTED: N-acylneuraminate cytidylyltransferase	1.96
741946731	PREDICTED: ankyrin repeat domain-containing protein 26-like isoform X2	0.35

62460494	hemoglobin fetal subunit beta	0.56
84000167	WD repeat-containing protein 61	1.36
741960002	PREDICTED: protein arginine N-methyltransferase 1 isoform X2	0.76
297483902	PREDICTED: apolipoprotein R	0.61
155372051	tropomyosin alpha-4 chain	0.83
78369240	U6 snRNA-associated Sm-like protein LSm4	0.7
122692397	latexin	0.79
77735445	protein mago nashi homolog	1.22
741939300	PREDICTED: dual specificity mitogen-activated protein kinase kinase 1 isoform X1	1.21
528995215	PREDICTED: dual specificity mitogen-activated protein kinase kinase 4 isoform X2	1.21
741898851	PREDICTED: MAP/microtubule affinity-regulating kinase 3 isoform X1, partial	0.83
528957564	PREDICTED: methionine adenosyltransferase 2 subunit beta isoform X1	0.81
528966905	PREDICTED: protein max isoform X2	0.76
741957547	PREDICTED: mediator of RNA polymerase II transcription subunit 15 isoform X3	0.79
300794942	DNA mismatch repair protein Msh6	0.74
27806841	interferon-induced GTP-binding protein Mx1	0.73
528936325	PREDICTED: N-alpha-acetyltransferase 50 isoform X1	1.3
375065860	NAD kinase 2, mitochondrial	1.29
300795748	NEDD8-activating enzyme E1 regulatory subunit	0.77
331284195	nucleolin	1.38
78369204	protein NDRG2	1.24
28372495	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11	1.23
75812936	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial precursor	1.32
28603776	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial precursor	0.72
528944090	PREDICTED: nexilin isoform X5	0.76
300794221	nuclear protein localization protein 4 homolog	0.82
83035119	nuclear transport factor 2	0.79
741958202	PREDICTED: prolyl 3-hydroxylase OGFOD1 isoform X1	0.65
27807193	platelet-activating factor acetylhydrolase IB subunit beta	1.27

75812940	phosphatidylethanolamine-binding protein 1	1.27
528913445	PREDICTED: presequence protease, mitochondrial isoform X2	1.21
329664500	pyruvate kinase PKM	1.25
528961976	PREDICTED: pyruvate kinase PKM isoform X1	1.26
741932605	PREDICTED: perilipin-3 isoform X3	0.82
116004039	peptidyl-prolyl cis-trans isomerase C precursor	1.22
741957590	PREDICTED: protein phosphatase 1F	1.53
115497768	relA-associated inhibitor	1.24
528943961	PREDICTED: cAMP-dependent protein kinase catalytic subunit beta isoform X8	1.22
741948151	PREDICTED: pre-mRNA-processing factor 6 isoform X1	0.73
115496548	proteasome assembly chaperone 1	1.26
741926509	PREDICTED: prostaglandin E synthase 3 isoform X1	0.83
157428086	ras-related protein Rab-8A	0.8
77736231	ras-related protein Ral-A	1.2
56118252	RING finger protein 113A	1.27
741937627	PREDICTED: ribosome production factor 2 homolog isoform X1	0.77
27807465	60S ribosomal protein L10	0.79
62751646	60S ribosomal protein L13	0.7
116004215	60S ribosomal protein L13a	0.81
118150852	60S ribosomal protein L15	0.7
62751887	60S ribosomal protein L26	0.77
77404275	60S ribosomal protein L27	0.79
77735585	60S ribosomal protein L36a	0.8
62460480	60S ribosomal protein L4	0.74
114053031	39S ribosomal protein L48, mitochondrial precursor	0.81
72534798	60S ribosomal protein L6	0.72
62460552	60S ribosomal protein L7	0.77
77736197	60S ribosomal protein L8	0.78
164420694	60S ribosomal protein L9	1.21
70778762	60S acidic ribosomal protein P1	1.2
66792924	40S ribosomal protein S11	0.76
77735975	28S ribosomal protein S26, mitochondrial precursor	1.23
528994013	PREDICTED: 28S ribosomal protein S23, mitochondrial isoform X3	1.23
27807381	40S ribosomal protein S29	1.23

70778956	40S ribosomal protein S8	0.79
155372029	40S ribosomal protein S9	0.69
741947465	PREDICTED: ribosome-binding protein 1 isoform X2	1.31
300798287	sec1 family domain-containing protein 1	0.83
115497454	protein SEC13 homolog	1.28
300794266	SEC23-interacting protein	1.3
741978352	PREDICTED: protein transport protein Sec24C isoform X2	1.31
115497008	protein transport protein Sec61 subunit beta	0.82
70778796	splicing factor 3B subunit 5	1.3
77736509	S-phase kinase-associated protein 1	1.35
82617542	monocarboxylate transporter 1	1.24
288557348	SWI/SNF complex subunit SMARCC2	0.8
115496404	U1 small nuclear ribonucleoprotein C	0.78
329664862	S1 RNA-binding domain-containing protein 1	0.73
741921253	PREDICTED: serine/arginine-rich splicing factor 11 isoform X4	1.49
329664840	synaptopodin	0.82
84000143	T-complex protein 1 subunit alpha	1.21
114051768	tudor domain-containing protein 3	1.36
300797062	tudor domain-containing protein 6	1.52
529000498	PREDICTED: THUMP domain-containing protein 3 isoform X1	1.3
114326224	tight junction protein ZO-3	1.25
300794719	E3 ubiquitin-protein ligase TRIP12	0.78
27806789	transthyretin precursor	1.29
529005013	PREDICTED: thioredoxin-like protein 1 isoform X2	1.21
83035103	ubiquitin-conjugating enzyme E2 H	1.24
528979920	PREDICTED: ubiquitin conjugation factor E4 B isoform X1	1.22
114050863	ubiquitin-like domain-containing CTD phosphatase 1	0.72
529012185	PREDICTED: UBX domain-containing protein 1 isoform X1	0.78
62751620	ubiquitin-fold modifier-conjugating enzyme 1	0.82
529006388	PREDICTED: ubiquitin carboxyl-terminal hydrolase 7 isoform X2	1.37
115496338	vesicle-associated membrane protein-associated protein A	1.2
78369492	vacuolar protein sorting-associated protein 28 homolog	0.79
78045497	vitronectin precursor	0.43
741916372	PREDICTED: xin actin-binding repeat-containing protein 2 isoform X2	0.72

126723764	cap-specific mRNA (nucleoside-2'-O-)-methyltransferase 1	0.8
78042540	synaptobrevin homolog YKT6	0.82
148224064	transcriptional repressor protein YY1	0.76
84370039	zinc finger protein ZPR1	0.77
528942220	PREDICTED: rho guanine nucleotide exchange factor 2 isoform X5	1.2
528962021	PREDICTED: geranylgeranyl transferase type-2 subunit alpha isoform X1	0.83
528979380	PREDICTED: glyoxylate reductase/hydroxypyruvate reductase	1.35

Primers of the selected genes for qRT-PCR and their relative expression.

2 **Table 3. Gene ontology analysis of differentially expressed proteins in VPS28 knockdown**

3 **BMECs.**

GO ID	Term	*P* Value
	Biological Process	
GO:0002181	cytoplasmic translation	3.60E-07
GO: 0006412	translation	8.90E-07
GO:0042632	cholesterol homeostasis	2.20E-03
GO:0033344	cholesterol efflux	2.70E-03
GO:0010873	positive regulation of cholesterol esterification	2.80E-03
GO:0034380	high-density lipoprotein particle assembly	3.70E-03
GO:0000463	maturation of LSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)	4.70E-03
GO:0043691	reverse cholesterol transport	7.10E-03
GO:0033700	phospholipid efflux	8.40E-03
GO:0098779	mitophagy in response to mitochondrial depolarization	1.00E-02
GO:0042157	lipoprotein metabolic process	1.30E-02
GO:0019433	triglyceride catabolic process	1.50E-02
GO:0006904	vesicle docking involved in exocytosis	2.30E-02
GO:0000027	ribosomal large subunit assembly	2.30E-02
GO:0018158	protein oxidation	2.30E-02
GO:0006403	RNA localization	2.30E-02
GO:0010628	positive regulation of gene expression	2.60E-02
GO:0001843	neural tube closure	3.10E-02
GO:0006695	cholesterol biosynthetic process	3.20E-02
GO:0051028	mRNA transport	3.20E-02
GO:0042921	glucocorticoid receptor signaling pathway	3.50E-02
GO:0010903	negative regulation of very-low-density lipoprotein particle remodeling	3.50E-02
GO:0006046	N-acetylglucosamine catabolic process	3.50E-02
GO:1901998	toxin transport	3.70E-02
GO:0006888	ER to Golgi vesicle-mediated transport	3.80E-02
GO:0018206	peptidyl-methionine modification	4.60E-02
GO:0042159	lipoprotein catabolic process	4.60E-02
GO:0042158	lipoprotein biosynthetic process	4.60E-02
	Cellular Component	
GO:0022625	cytosolic large ribosomal subunit	4.00E-12
GO:0070062	extracellular exosome	2.70E-08
GO:0005925	focal adhesion	1.30E-05
GO:0016020	membrane	1.70E-04
GO:0034361	very-low-density lipoprotein particle	3.60E-04
GO:0005840	ribosome	8.30E-04

GO:0022627	cytosolic small ribosomal subunit	9.90E-04
GO:0042627	chylomicron	3.30E-03
GO:0072562	blood microparticle	9.70E-03
GO:0034363	intermediate-density lipoprotein particle	3.30E-02
GO:0005730	nucleolus	3.80E-02
GO:0008180	COP9 signalosome	4.60E-02
GO:0005737	cytoplasm	4.70E-02
	Molecular Function	
GO:0003735	structural constituent of ribosome	5.20E-10
GO:0003723	poly(A) RNA binding	2.90E-09
GO:0017127	cholesterol transporter activity	3.30E-04
GO:0019843	rRNA binding	5.20E-04
GO:0060228	phosphatidylcholine-sterol O-acyltransferase activator activity	1.40E-03
GO:0003729	mRNA binding	6.90E-03
GO:0005543	phospholipid binding	1.70E-02
GO:0003743	translation initiation factor activity	2.10E-02
GO:0003723	RNA binding	3.50E-02
GO:0008035	high-density lipoprotein particle binding	3.50E-02
GO:0070653	high-density lipoprotein particle receptor binding	3.50E-02
GO:0015485	cholesterol binding	4.60E-02