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1 **Attenuated metabolism is a hallmark of obesity as revealed by**
2 **comparative proteomic analysis of human omental adipose tissue**

3

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20 ABSTRACT

21 Obesity is recognized as an epidemic health problem worldwide. In humans, the accumulation
22 of omental rather than subcutaneous fat appears to be tightly linked to insulin resistance, type 2
23 diabetes and cardiovascular disease. Differences in gene expression profiles in the adipose
24 tissue comparing non-obese and obese subjects have been well documented. However, to date,
25 no comparative proteomic studies based on omental fat have investigated the influence of
26 obesity in protein expression. In this work, we searched for proteins differentially expressed in
27 the omental fat of non-obese and obese subjects using 2D-DIGE and MS. Forty-four proteins,
28 several of which were further studied by immunoblotting and immunostaining analyses, showed
29 significant differences in the expression levels in the two groups of subjects. Our findings reveal
30 a clearly distinctive proteomic profile between obese and non-obese subjects which emphasizes:
31 i) reduced metabolic activity in the obese fat, since most down-regulated proteins were engaged
32 in metabolic pathways; and ii) morphological and structural cell changes in the obese fat, as
33 revealed by the functions exerted by most up-regulated proteins. Interestingly, transketolase and
34 aminoacylase-1 represent newly described molecules involved in the pathophysiology of
35 obesity, thus opening up new possibilities in the study of obesity.

36

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38 Keywords: 2D-DIGE, MALDI-MS, obesity, human adipose tissue, omental fat, TKT, ACY-1,
39 comparative proteomic study, metabolic pathways

40

41 **1. Introduction**

42

43 Obesity is one of the most important public health problems facing the world today and has
44 increased dramatically over the last decades in children and adolescents [1]. Obese subjects
45 suffer from decreased life quality and expectancy as well as increased risk of suffering insulin
46 resistance, type 2 diabetes, cardiovascular disease (CVD), hepatic steatosis, pulmonary and
47 muscular pathologies, psychological disorders and cancer, among others [2]. A person's weight
48 and body composition are likely determined by interaction between his/her genetic makeup and
49 social, cultural, behavioral, and environmental factors. The intake of energy-dense foods,
50 especially when combined with reduced physical activity, is very likely to contribute to the high
51 prevalence of obesity; however, the existence of complex systems that regulate energy balance
52 calls for a broader view of this paradigm [3].

53 In humans, the adipose tissue is dispersed throughout the body with major intra-abdominal
54 depots around the omentum, intestines, and perirenal areas, as well as in subcutaneous depots in
55 the buttocks, thighs, and abdomen. These two fat depots, the subcutaneous and the omental fat,
56 exhibit unique biochemical and cellular properties, such as response to sex hormones, and
57 different secretion profiles [4], including a different lipolytic program [5]. Moreover, the
58 omental, but not the subcutaneous, fat drains directly into the portal circulation, and some data
59 point out to excessive free fatty acid release from the omental adipose tissue in central obesity
60 [6]. In fact, it is well established that the size of the omental, more than the subcutaneous, fat is
61 strongly related to a higher risk of obesity-related co-morbidities, including insulin resistance,
62 type 2 diabetes, dyslipidemia and CVD. As a consequence of extensive recent investigation, the
63 adipose tissue is no longer regarded a mere fat reservoir, but an endocrine organ which cross-
64 talks with other essential organs like the liver, the muscle, the pancreas, and the brain, being a
65 crucial regulator of whole-body homeostasis.

66 Gene expression studies (i.e. microarrays and RT-PCR) using adipose tissue from obese and
67 non-obese subjects have yielded important insights into the pathogenesis of obesity and related
68 diseases, (reviewed in [7]). Results pointed out that: i) obesity represents a chronic

69 inflammatory condition, since genes related with inflammation are up-regulated in response to
70 obesity; ii) the differentiation state of obese adipocytes is altered; and iii) the expression of
71 adipogenic genes is decreased in obesity [8-11]. As far as the latter is concerned, it has been
72 suggested that the limited lipogenic and/or adipogenic capacity of obese adipocytes might lead
73 to spillover of excess lipids to other tissues, and lipotoxicity could contribute to the
74 pathogenesis of type 2 diabetes [12].

75 At the protein level the knowledge about human adipose tissue is limited. A very few proteomic
76 studies have been published using either whole adipose tissue or isolated cells from both fat
77 depots (reviewed in [13]). The majority of these works have studied human adipogenesis or
78 adipose tissue secretome. Recently, however, our group and others have resorted to 2D-DIGE
79 and MS to explore the differences between omental and subcutaneous fat [14-16]. Despite of
80 some drawbacks inherent to 2-DE analysis (mainly the poor representation of low-abundant or
81 very hydrophobic proteins as well as those with extreme pI and molecular weight), the
82 quantitative comparison of proteins in two or more conditions based on 2D-DIGE/MS is a
83 widespread, robust methodology to assess differential protein expression. This approach
84 provides great analytical precision, dynamic range and sensitivity, therefore allowing a
85 reproducible and reliable differential analysis [17]. Nevertheless, to date, no proteomic studies
86 based on omental fat have investigated the influence of obesity in protein expression, given that
87 the omental adipose tissue has been tightly linked to obesity-associated co-morbidities. In this
88 work, we have compared for the first time the omental fat from non-obese and morbidly obese
89 subjects by 2D-DIGE and MS, revealing 44 modulated proteins in response to obesity. Our
90 findings emphasize a noticeably decreased expression of proteins related to metabolic processes
91 in response to obesity together with a down-regulation of mitochondrial enzymes, which is
92 consistent with a reduced metabolic activity in the obese adipose tissue. In addition, most of the
93 proteins found up-regulated in obesity develop structural functions in the cell, which account for
94 the morphological changes undergone by obese adipocytes. Therefore, our results support a
95 neatly distinctive biological profile in the omental fat of obese and non-obese subjects regarding
96 protein expression.

97 **2. Materials and methods**

98

99 2.1. Study design

100 Omental fat from morbidly obese ($n=6$) and non-obese ($n=6$) subjects obtained during surgery
101 were analyzed by a proteomic approach using 2D-DIGE. Samples were labelled using
102 fluorophore dye-swapping to avoid labelling bias, combined in pairs and separated by
103 electrophoresis. Image analysis revealed modulated proteins which were identified by MS.
104 Results validation was performed by Western Blot with an additional set of subjects.
105 Immunostaining assays were performed to study selected proteins not only in adipose tissue
106 samples, but also in human omental adipocyte cultures. The 3T3-L1 cell line was used to study
107 whether these proteins were modulated in the adipocyte differentiation process.

108

109 2.2. Biological samples

110 Omental adipose tissue samples were obtained from 26 women, including 12 non-obese and 14
111 morbidly obese. Non-obese subjects had a body mass index (BMI) $< 30 \text{ kg/m}^2$ (BMI ranged
112 from 22.1 to 28.3 Kg/m^2), and age ranged from 25 to 56 years. Morbidly obese subjects had a
113 BMI $> 40 \text{ kg/m}^2$ (BMI ranged from 40.7 to 48.5 Kg/m^2), and age ranged from 39 to 58 years.
114 All these subjects had been submitted for elective surgical procedures (cholecystectomy,
115 surgery of abdominal hernia and gastric by-pass surgery). During surgery, biopsies of adipose
116 tissues were obtained after an overnight fast, washed in chilled 9 g/L NaCl solution, partitioned
117 into pieces, and immediately frozen in liquid nitrogen and stored at -80°C until protein
118 extraction. The surgeon aimed to obtain the samples from similar anatomical locations in all the
119 subjects. All women were of Caucasian origin and reported that their body weight had been
120 stable for at least three months before the study. None of the subjects had type 2 diabetes or any
121 other systemic disease apart from obesity and all were free of any infections within the previous
122 month before the study. Liver disease and thyroid dysfunction were specifically excluded by
123 biochemical work-up. Other exclusion criteria for those patients included the following: 1)
124 clinically significant hepatic, neurological, or other major systemic disease, including

125 malignancy; 2) history of drug or alcohol abuse, defined as >80 g/day, or serum transaminase
126 activity more than twice the upper normal range limit; 3) elevated serum creatinine
127 concentrations; 4) acute major cardiovascular event in the previous 6 months; 5) acute illnesses
128 and current evidence of chronic inflammatory or infectious diseases; and 6) mental illness
129 rendering the subjects unable to understand the nature, scope, and possible consequences of the
130 analysis. The study was conducted according to the recommendations of the Declaration of
131 Helsinki and was approved by the ethics committees of Hospital Dr. Josep Trueta (Girona,
132 Spain). Signed informed consent was obtained from all subjects.

133

134 2.3. 2D-DIGE analysis

135 Proteins were extracted from omental adipose tissue biopsies (100 mg) by using the 2D
136 Grinding Kit (GE Healthcare, Uppsala, Sweden) in Lysis Buffer (7 M urea, 2 M thiourea, 4%
137 CHAPS, and 30 mM Tris-HCl pH 8.5) containing 50 mM DTT. The extract was shaken for 30
138 min at room temperature and centrifuged at 15000xg for 30 minutes. Proteins were precipitated
139 with the 2D-CleanUp Kit (GE Healthcare) and redissolved in Lysis Buffer. The protein
140 concentration was determined using RC/DC Protein Assay (Bio-Rad Laboratories, Hercules,
141 CA, USA). Proteins were labelled according to the manufacturer's instruction (GE Healthcare).
142 Briefly, 50 µg of adipose tissue protein extracts were minimally labeled with 400 pmol of the *N*-
143 hydroxysuccinimide esters of Cy3 or Cy5 fluorescent cyanine dyes on ice in the dark for 30 min.
144 All experiments comprised an internal standard containing equal amounts of each cell lysate,
145 which was labelled with Cy2 dye. The labelling reaction was quenched with 1 µl of 10 mM
146 lysine on ice in the dark for 10 min. The internal standard and the individual omental fat extracts
147 from non-obese and obese subjects were combined and run in a single gel (150 µg total
148 proteins). Proteins extracts were diluted in Rehydration Buffer (7 M urea, 2 M thiourea, 2%
149 CHAPS, 0.8% (v/v) IPG buffer 3-11NL), reduced with 50 mM DTT, and applied by cup-
150 loading to 24 cm IPG strips pH 3-11NL, which were previously rehydrated with Rehydration
151 Buffer containing 100 mM hydroxyethyl disulfide (DeStreak, GE Healthcare). The first and

152 second dimension together with the equilibration step were performed following the procedure
153 previously described [14].

154

155 2.4. Image acquisition and analysis

156 After SDS-PAGE, gels were scanned with a Typhoon 4100 scanner (GE Healthcare) at 100 μ m
157 resolution using appropriate individual excitation and emission wavelengths, filters and
158 photomultiplier (PMT) sensitivity for each Cy2, Cy3 and Cy5 dyes (PMT values: 510, 510 and
159 475 respectively). Gel images were analyzed with the DIA (Differential in-gel Analysis) module
160 of the DeCyder v7 software (GE Healthcare) for automatic spot detection, background
161 subtraction, quantification and normalization with low experimental variation (DeCyder
162 Differential Analysis Software User Manual, version 7; GE Healthcare, 2009). The Biological
163 Variation Analysis (BVA) module utilized those images individually processed with the DIA
164 module to match protein spots across gels, using the internal standard for gel-to-gel matching.
165 Statistical analysis was then carried out to determine protein expression changes. P values lower
166 than 0.05 as calculated from Student's t test were considered significant. Multivariate analysis
167 was performed by Principal Components Analysis (PCA) using the algorithm included in the
168 Extended Data Analysis (EDA) module of the DeCyder software based on the spots matched
169 across all gels.

170

171 2.5. In-gel trypsin digestion and mass spectrometry

172 Protein spots showing significantly altered expression levels in the 2 groups of samples by
173 DeCyder Software were selected for gel excision from silver-stained gels, digested
174 automatically on a Proteineer DP robot (Bruker Daltonik, Bremen, Germany) using the protocol
175 of [18] and analyzed in an Ultraflex MALDI TOF/TOF mass spectrometer (Bruker Daltonik)
176 [19] to obtain the corresponding MALDI-MS and MALDI-MS/MS spectra. In a first step,
177 MALDI-MS spectra were acquired by averaging 300 individual spectra in the positive ion
178 reflector mode at 50 Hz laser frequency in a mass range from 800 to 4000 Da. In a second step,
179 precursor ions showing in the MALDI-MS mass spectrum were subjected to fragment ion

180 analysis in the tandem (MS/MS) mode to average 1000 spectra. Peak labelling, internal
181 calibration based on two trypsin autolysis ions with $m/z = 842.510$ and $m/z = 2211.105$, as well
182 as removal of known trypsin and keratin peptide masses were performed automatically using the
183 flexAnalysis 2.2 software (Bruker Daltonik). No smoothing or any further spectral processing
184 was applied. MALDI-MS and MS/MS spectra were manually inspected in detail and reacquired,
185 recalibrated and/or relabelled using the aforementioned programs and homemade software when
186 necessary.

187

188 2.6. Database searching

189 MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker
190 Daltonik) to search a nonredundant protein database (NCBI nr 20091022; $\sim 7.0 \times 10^6$ entries;
191 National Centre for Biotechnology Information, Bethesda, USA), using the Mascot 2.2 software
192 (Matrix Science, London, UK; <http://www.matrixscience.com>) [20]. Other relevant search
193 parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (C);
194 allow up to 1 missed cleavage; peptide tolerance ± 20 ppm; MS/MS tolerance ± 0.5 Da. Protein
195 scores greater than 81 were considered significant ($p < 0.05$).

196

197 2.7. Cell culture and adipocyte differentiation

198 Isolated human omental pre-adipocytes (Zen-Bio, Inc. Raleigh, NC, USA) were cultured with
199 omental pre-adipocytes medium (Zen-Bio, Inc.) at 37°C and 5% CO_2 and differentiated using
200 omental differentiation medium (Zen-Bio, Inc.) according to the method outlined by Ortega et al
201 [21]. Two weeks after the initiation of differentiation, cells appeared rounded with large lipid
202 droplets in the cytoplasm and were considered mature adipocytes. Murine 3T3-L1 fibroblasts
203 (CCL 92.1, American Type Culture Collection) were grown to confluence in DMEM containing
204 10% calf serum. The differentiation to adipocytes was induced according to the procedure
205 described by Ortega et al [21]. On days 0, 3, 5 and 9, three replicated cell samples were
206 separately collected for later immunoassays.

207

208 2.8. Immunoblotting analysis

209 Fat tissue or cultured cells were homogenized in radioimmuno precipitation assay (RIPA)
210 buffer as described in [14]. Protein extracts (*ca.* 10 µg) were loaded, resolved on SDS-PAGE
211 and transferred to Hybond ECL nitrocellulose membranes by conventional procedures.
212 Membranes were stained with 0.15% Ponceau red (Sigma-Aldrich, St Louis, MO, USA) to
213 ensure equal loading after transfer and then blocked with 5% (w/v) BSA or dried nonfat milk in
214 TBS buffer with 0.1% Tween 20. The antibodies used for Western Blot analysis revealed in
215 each case single bands at the expected molecular masses. The primary antibodies used were:
216 1:2000 rabbit anti-TKT (HPA029480), and 1:2000 rabbit anti-ACY-1 (A6609) (Sigma-
217 Aldrich); 1:2000 goat anti-Beta-actin (sc-1616); 1:4000 rabbit anti-SPHK1 (sc-48825), and
218 1:200 goat anti-FABP5 (sc-16060) (Santa Cruz Biotechnology); 1:1000 mouse anti-HSP70
219 (C92F3A-5) (Stressgen Bioreagents); 1:500 rabbit anti-FABP4 (Eurogentec, Seraing, Belgium).
220 Blots were incubated with the appropriate IgG-HRP-conjugated secondary antibody.
221 Immunoreactive bands were visualized with ECL-plus reagent kit (GE Healthcare). Blots were
222 exposed for different times; exposures in the linear range of signal were selected for
223 densitometric evaluation. Optical densities of the immunoreactive bands were measured using
224 Image J analysis software. Statistical comparisons of the densitometry data were carried out
225 using the Student's *t* test for samples, and results were expressed as means ± standard deviation
226 (SD) using SPSS 16.0 (SPSS Inc., Illinois, USA). Statistical significance was set at $p < 0.05$.

227

228 2.9. Immunohistochemistry

229 Five-micron sections of formalin-fixed paraffin-embedded adipose tissue were deparaffinised
230 and rehydrated prior to antigen unmasking by boiling in 1 mM EDTA, pH 8. Sections were
231 blocked in normal serum and incubated overnight with rabbit anti-TKT (1:500 dilution) or
232 rabbit anti-ACY-1 (1:200 dilution) antibodies. Secondary antibody staining was performed
233 using the VECTASTAIN ABC kit (Vector Laboratories, Inc. Burlingame, CA) and detected
234 with diaminobenzidine (DAB, Vector Laboratories, Inc.). Sections were counterstained with
235 hematoxylin prior to dehydration and coverslip placement, and examined under a Nikon Eclipse

236 90i microscope. As a negative control, the procedure was performed in the absence of primary
237 antibody.

238

239 2.10. Immunofluorescence

240 Frozen adipose tissue sections or cultured cells were fixed with 4% paraformaldehyde and
241 permeabilized for 30 min with 0.1% Triton X-100 in PBS. Staining was performed overnight at
242 4°C with rabbit anti-TKT (1:500 dilution) or with rabbit anti-ACY-1 (1:400 dilution) antibodies,
243 washed, and visualized using Alexa Fluor 546 goat anti-rabbit antibody (1:500; Molecular
244 Probes Inc., OR, USA). The slides were counterstained with DAPI (4,6-diamidino-2-
245 phenylindole) to reveal nuclei. The lipophilic fluorescence dye BODIPY 493/503 was used for
246 lipid droplet labelling according to the manufacturer's instruction (Molecular Probes Inc.). The
247 slides were examined under a Leica TCS SP5 fluorescent microscope (Heidelberg, Germany).
248 As a negative control, the assay was performed in the absence of primary antibody.

249

250 **3. Results**

251

252 3.1. Proteomic analysis of obese and non-obese adipose tissue samples

253 To detect proteins differentially expressed in obesity, omental fat samples from morbidly obese
254 ($n=6$) and non-obese ($n=6$) females were analyzed by 2D-DIGE. Protein extracts were labelled
255 using dye-swapping with either Cy3 or Cy5 fluorescent dye to avoid labelling bias arising from
256 the fluorescence properties of gels at different wavelengths. Then each Cy3/Cy5-labelled
257 sample pair was mixed with a Cy2-labelled internal standard and loaded onto each gel. After 2-
258 DE, the Cy2, Cy3 and Cy5 channels were individually imaged from each gel (Supplemental Fig.
259 1). Automated image analysis performed with DeCyder software detected approximately 2700
260 spots per gel in the 3-11 NL pH range with a molecular mass of 10-150 kDa, of which 1200
261 spots were matched throughout all gels. Multivariate PCA showed that the "non-obese group"
262 was efficiently discriminated from the "obese group" (data not shown). DeCyder statistical
263 analyses showed that 70 protein spots were differentially expressed at $p<0.05$ considering only

264 those spots present in all gels. These spots were excised from silver-stained gels, digested with
265 trypsin, and analyzed by MALDI-MS followed by database search. Fifty-six spots, which
266 corresponded to 44 unique proteins could be identified (Fig. 1 and Table 1). Twenty proteins
267 were increased and 24 decreased in response to obesity.

268

269 3.2. Functional classification of the proteins differentially expressed

270 To understand the biological relevance of protein expression changes in response to obesity, the
271 Protein Analysis Through Evolutionary Relationship (PANTHER) application
272 (<http://www.pantherdb.org/>) was used. This classification system uses information on protein
273 sequence to assign a gene to an ontology group on the basis of the Gene Ontology (GO) terms
274 <http://www.geneontology.org/>. Thus, the two sets of up- and down-regulated proteins were
275 searched for significantly over-represented ($p < 0.05$) GO terms. Two key Biological Process
276 classes were found significantly enriched in the group of down-regulated proteins: *Metabolic*
277 *Process* and *Generation of Precursor Metabolites and Energy*, while three key Biological
278 Process classes (*Cellular Process*, *Developmental Process* and *Cellular Component*
279 *Organization*) were significantly enriched in the set of up-regulated proteins. Likewise, the
280 categorization based on the Molecular Function GO category showed that most down-regulated
281 proteins accounted for one key significant class, *Catalytic Activity*, while *Structural Molecule*
282 *Activity* revealed as the unique key GO term with significant enrichment in the up-regulated
283 proteins from obese adipose tissue (Fig. 2).

284 In addition, PANTHER application mapped the 44 differentially expressed proteins into parent
285 and child categories with regard to their Molecular Function and Biological Process GO terms
286 (Supplementary Table 2), highlighting that most of the down-regulated proteins were engaged
287 in metabolic pathways. Our results have also revealed that the set of down-regulated proteins
288 comprised numerous (14 out of 24, 58%) mitochondrial enzymes, overall supporting a reduced
289 metabolic activity in the obese adipose tissue.

290

291 3.3. Validation of differential protein expression

292 **Western Blot** analyses were performed in an additional set of non-obese and morbidly obese
293 women for two proteins whose expression in human adipose tissue had not been previously
294 documented, TKT and ACY-1, together with two molecules, HSP70 and FABP5 that had been
295 earlier studied in fat and/or in obesity and related co-morbidities. One of these proteins was
296 shown up-regulated (HSP70) and the other three were found down-regulated (TKT, ACY-1 and
297 FABP5) in response to obesity. Immunoblotting analysis using an antibody against TKT
298 confirmed that this protein was over-expressed ($p<0.05$) in the non-obese group of subjects,
299 confirming 2-DE findings (Fig. 3). Likewise, ACY-1 levels were significantly more abundant
300 ($p<0.05$) in the non-obese subjects (Fig. 3), in agreement with 2D-DIGE results. Both TKT and
301 ACY-1 were studied by immunostaining methods, as well as in the adipocyte differentiation
302 process. Immunoblotting analysis revealed that HSP70 was significantly increased in obese vs.
303 non-obese individuals ($p<0.05$), thus confirming 2D-DIGE results (Fig. 3). By using an
304 antibody anti-FABP5, immunoblotting analysis revealed an over-expression of FABP5 protein
305 in the omental adipose tissue from non-obese compared to obese subjects; however this result
306 did not reach statistical significance ($p=0.06$) mostly due to the high SD observed in non-obese
307 samples (data not shown). It must be noted that **Western Blot** assay may fail to validate
308 particular protein isoforms found differentially expressed by 2D-DIGE/MS as they rely on
309 antibodies lacking the necessary specificity.

310

311 3.4. Immunostaining analyses

312 Immunohistochemical and immunofluorescence approaches were performed to determine the
313 cellular distribution of TKT and ACY-1 proteins in biopsies of omental fat given that, as far as
314 we know, these proteins have not been earlier analysed in this tissue. TKT was assayed in
315 sections of omental adipose tissue by both techniques revealing similar results.
316 Immunofluorescence detection showed a bright staining pattern mainly in the cytoplasm of
317 adipocytes and of stromal-vascular fraction (SVF) cells, as well as in the nuclei of a few cells
318 (Fig. 4A). To determine whether the stained nuclei pertained to adipocytes,
319 immunofluorescence analysis from a cellular culture of human pre-adipocytes and differentiated

320 adipocytes was performed. This analysis showed that in pre-adipocytes TKT was localized in
321 the cytoplasm as well as in the nucleus, while in adipocytes only the cytoplasm but not the
322 nucleus was stained (Fig. 4B and 4C). TKT expression was also confirmed in adipose tissue
323 macrophages by co-staining assays using CD68 (not shown).

324 ACY-1 was also assayed in sections of omental fat by immunostaining analyses, which showed
325 that ACY-1 was expressed in the cytoplasm as well as in the nucleus of adipocytes and SVF
326 cells, including omental mesothelial cells (Supplemental Fig. 2A and 2B). Immunofluorescence
327 analysis revealed the presence of ACY-1 in the nucleus of cultured human omental pre-
328 adipocytes (Fig. 5A), while in differentiated adipocytes ACY-1 localized around cytosolic lipid
329 droplets and, to a lesser extent, in the nucleus (Fig. 5B and 5C). In addition, we had also
330 performed immunofluorescence analysis in 3T3-L1 cells during the adipogenic process. As
331 illustrated in Supplemental Fig. 3A, ACY-1 was localized exclusively in 3T3-L1 fibroblast
332 nuclei (day 0), as was the case for human pre-adipocytes; however, in 3T3-L1 differentiated
333 adipocytes (day 9), ACY-1 was shown around lipid droplets in the cytoplasm, as well as in the
334 majority of the nuclei (Supplemental Fig. 3B and 3C).

335

336 3.5. 3T3-L1 adipogenesis

337 To further study TKT and ACY-1 proteins, we performed immunoblotting analysis with
338 proteins extracted during the adipogenic maturation of 3T3-L1 cells using the above-described
339 specific antibodies. TKT and ACY-1 were significantly augmented with adipocyte
340 differentiation in parallel to the expression of FABP4, which was used as an adipogenesis
341 control (Fig. 6).

342

343 **4. Discussion**

344 Over the last years an increasing number of studies have focused in the analysis of gene
345 expression to gain insight into obesity and related pathologies. However only a few number of
346 studies have resorted to proteomic methods to identify human fat proteins associated to these
347 disorders. In the present study we have employed a proteomic approach based on 2D-DIGE and

348 MALDI-MS to uncover differences in protein expression using biopsies of omental fat from
349 non-obese and morbidly obese individuals, reporting for the first time a set of 44 proteins that
350 are significantly modulated in these two sets of subjects. Our study has focused on omental
351 adipose tissue as this fat depot has been long associated with augmented risk of suffering
352 pathologies related to obesity [22].

353 The down-regulation of proteins related to metabolic processes such as *Amino Acid Metabolism*,
354 *Carbohydrate Metabolism* and *Lipid Metabolism* suggests a reduction of metabolic activity in
355 the obese omental fat, and is consistent with previous mRNA studies [8-10, 23]; thus, Ortega *et*
356 *al.* [10] demonstrated the down-regulation of the main lipogenic enzymes in obese omental fat
357 using a large cohort of individuals. In this scenario, these findings provide evidence for an
358 impaired capacity of the adipose tissue to function as an energy reservoir. In addition, it is
359 noteworthy the high number of mitochondrial enzymes included in the set of down-regulated
360 proteins in the obese adipose tissue, 14 proteins out of 24, which is consistent with the reduction
361 in the oxidative metabolism in obesity. Our findings are in agreement with previous microarray
362 analysis revealing a coordinated down-regulation of catabolic pathways operating in the
363 mitochondria such as: fatty acid β oxidation, tricarboxylic acid cycle and electron transport
364 chain [24]. In addition, a strong correlation between impaired adipocyte mitochondrial activity
365 and/or content and obesity has been well documented [25-27]. Decreased mitochondrial
366 capacity in adipocytes may alter adipocyte insulin sensitivity and/or function due to the high
367 energetic requirements for fatty acid storage, adipokine secretion [28], insulin signalling [29],
368 and glucose uptake. Therefore, the relatively high number of mitochondrial proteins found
369 down-regulated in our study is consistent with previous evidences.

370 The set of up-regulated proteins, which pertain to the following significantly enriched classes:
371 *Cellular Process*, *Developmental Process*, *Cellular Component Organization*, and *Structural*
372 *Molecule Activity*, suggests that the enlargement of the obese adipocytes, by increasing fat
373 storage, is accompanied by: i) cytoskeleton changes, such as alteration of LMNA, LMNB1 and
374 integrin alpha 7; ii) changes in the extracellular matrix (ECM), such as collagen (COL6A3) and
375 lumican; and iii) tissue structure modifications, such as alteration in epithelial cytokeratins, CK-

376 7, CK-8 and CK-19, compatible with omental mesothelium changes. Our findings showing the
377 up-regulation of proteins controlling cell architecture and tissue remodelling are in agreement
378 with previous transcriptomic studies reporting that the expansion of adipose tissue is associated
379 with a remodelling of ECM together with changes of fat cell cytoskeleton [23, 30] compatible
380 with the need to adapt fat pads as adiposity increase.

381 Several relevant proteins highlighted by our study were more in-depth analyzed. Transketolase
382 (TKT) expression levels were reduced in obese patients. To our knowledge this is the first time
383 that a link between TKT and obesity is reported. This protein is a thiamine diphosphate (ThDP)-
384 dependent enzyme that catalyzes several reactions in the non-oxidative branch of the Pentose
385 Phosphate Pathway (PPP). In mammalian cells, the main function of PPP is to produce the
386 reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH), which functions in
387 detoxification processes and lipid biosynthesis. Another function of PPP is to convert hexose
388 into pentose, which is required for nucleic acid synthesis [31]. TKT haploinsufficient mice
389 showed a markedly reduction in adipose tissue (77%) [32] which could be induced by NADPH
390 deficiency, limiting the production of lipids in the fat. The reduced levels of TKT found in the
391 group of obese versus non-obese subjects could be explained by the occurrence of a
392 compensatory mechanism through which the obese adipose tissue would prevent further
393 enlargement. In this scenario, during the period of dynamic obesity large amounts of NADPH
394 are required for fatty acid biosynthesis, and an increase in TKT function is expected. In contrast,
395 it is well known that lipogenic pathways are reduced in established obesity [10] and TKT down-
396 regulation could be a late and adaptive process, aimed at limiting a further development of fat
397 mass. Immunostaining methods showed for the first time the expression of TKT in human
398 omental adipose tissue. TKT, an ubiquitous enzyme engaged in multiple metabolic pathways,
399 was widely distributed in adipocytes as well as other stromal cells. TKT was present mainly in
400 the cytoplasm of adipose cells, but a few nuclei also expressed the protein. Interestingly, the
401 nuclei of human pre-adipocytes expressed TKT in contrast to fully differentiated adipocytes, in
402 which TKT was only observed in the cytoplasm. A nuclear localization of TKT had already
403 been described [33]; in this regard, it is interesting to mention that in a highly proliferative state

404 increased cell division rate would require large amounts of phosphate pentose, which would
405 account for the nuclear localization of TKT. On the other hand, mature adipocytes keep pentose
406 phosphate consumption to a minimum while consuming many NADPH molecules for
407 lipogenesis, which would explain TKT localization in the cytoplasm of mature adipocytes.
408 Taken together, these findings highlight the potential role of this protein in adipose tissue and
409 adipogenesis.

410 2D-DIGE and immunoblotting analyses have shown significant down-regulation of
411 aminoacylase-1 (ACY-1) with obesity. ACY-1 is a cytosolic, homodimeric, zinc-binding
412 enzyme that function in the catabolism and retrieval of acylated amino acids. ACY-1 expression
413 has been found associated to renal carcinoma [34] and to an inborn metabolic disorder [35].
414 Nevertheless, this is the first report on ACY-1 expression in human fat. The nuclear localization
415 of ACY-1 is striking. In agreement with our finding, this enzyme had been previously found in
416 the nucleus of rat normal proximal tubular cells [34]. It is possible that several unidentified
417 nuclear proteins are substrates for ACY-1. It is noteworthy that ACY-1 physically interacts and
418 functionally modulates sphingosine kinase 1, SPHK1 [36], a lipid kinase that converts
419 sphingosine and ATP to sphingosine-1-phosphate (S1P). S1P is a potent signalling molecule
420 involved in angiogenesis and cell growth among other cellular processes [37]. Of note, we have
421 shown that both ACY-1 and SPHK1 are associated with adipogenesis in 3T3-L1 cells (Fig. 6
422 and Supplemental Fig. 4) as already found with the latter [38]. These results collectively support
423 the hypothesis that the SPHK1/ACY-1 system could play a role in obesity. Further studies are
424 underway to explore ACY-1 functional role in adipose tissue.

425 Our results based on the 3T3-L1 adipocytes differentiation process have shown for the first time
426 an increment of ACY-1 and TKT levels. Long-lasting fat excess has been evidenced to reduce
427 adipogenesis in adipose tissue to limit further expansion of fat mass [8-11]. We hypothesize that
428 the diminished levels of ACY-1 and TKT proteins in obesity stem from the impaired adipogenic
429 capacity of obese adipocytes.

430 The proteomic analysis has enabled the identification of other relevant proteins involved in
431 obesity or whose expression in fat has been widely documented. Results revealed the over-

432 expression in obese subjects of HSP70. HSPs not only serve as chaperones, mainly controlling
433 protein folding of newly translated polypeptides but also protect cells against many chronically
434 and acutely stressful conditions [39]. In spite of the numerous cellular processes in which
435 HSP70 takes part, it can be hypothesized that the higher levels of HSP70 found in the omental
436 fat from obese patients would serve to reduce the cellular stress associated to obesity. In skeletal
437 muscle, evidences have shown that there is a decreased expression of HSP70 in type 2 diabetes
438 patients [40, 41] and in mice models an elevation of HSP70 protected against obesity-induced
439 hyperglycemia, hyperinsulinemia, glucose intolerance and insulin resistance [41]. Nevertheless,
440 no similar studies have been conducted in adipose tissue to date. We performed Western Blot
441 analyses to compare omental fat samples from obese patients with and without type 2 diabetes.
442 Interestingly, in agreement with these evidences, our results revealed that the amount of HSP70
443 was significantly lower in type 2 diabetes obese subjects than in obese without type 2 diabetes
444 ($p=0.007$), as illustrated in Supplemental Fig. 5. Therefore this result supports a protection role
445 for HSP70.

446 It is well established that monoamine oxidase A (MAOA), a mitochondrial enzyme involved in
447 the oxidative deamination catabolism of neurotransmitters and exogenous amines, is highly
448 expressed in the adipocyte-enriched fraction of human adipose tissue [42]. Our results showed
449 reduced levels of MAOA in obese subjects, which is in agreement with an earlier report
450 revealing reduced MAOA activity in the adipose tissue from obese subjects [43]. Our study also
451 showed reduced expression levels of FABP5 in obese individuals, in consistence with previous
452 studies in subcutaneous fat [44]. FABP5 is a relevant adipose tissue protein that facilitates lipid
453 usage in metabolic pathways and plays a role in metabolic syndrome, insulin resistance, type 2
454 diabetes, and atherosclerosis, as elucidated in studies based on genetically modified mice [45].
455 Mice lacking FABP5 was protected against diet-induced obesity, insulin resistance and other
456 related diseases [46]. Ongoing studies in our laboratory attempt to evaluate whether these
457 results might be extrapolated to humans.

458

459 In summary, this work is, to our knowledge, the first proteomic study on omental fat comparing
460 non-obese and obese people and represents one of the few proteomic analyses in human adipose
461 tissue. Our findings evidence a clearly distinctive biological profile of obese and non-obese
462 subjects highlighting a noticeably decreased expression of proteins related to metabolic
463 processes and an increased expression of proteins that develop structural functions in the cell in
464 response to obesity. Besides, our study has revealed that TKT and ACY-1 are promising new
465 players involved in obesity. Our results will strengthen the understanding of molecular
466 pathogenesis of obesity, whilst the identified proteins can be regarded as potential targets for
467 future therapeutic strategies.

468

469

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477

478 **Appendix A. Supplementary data**

479 Supplementary data associated with this article contains Supplemental Fig. 1, 2, 3, 4 and 5,
480 Supplemental Table 1 and Supplemental Table 2, and can be found in the online version.

481

482

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612

613

614 **Figure Legends**

615

616 **Fig. 1.** Representative silver stained 2D gel of omental adipose tissue proteins using 24 cm pH3-
617 11NL (left to right) strips in the first dimension and 12% PAGE-SDS gels in the second
618 dimension. Numbers correspond to differentially expressed protein spots as indicated in Table
619 1. Supplemental Fig. 1 provides a more convenient visualization of the differential protein spots
620 on the silver stained 2D gel.

621

622 **Fig. 2.** Pie chart representations of PANTHER Biological Process and Molecular Function
623 classes significantly over-represented in the set of downregulated proteins (A, C) and in the set
624 of up-regulated proteins (B, D) in obesity. Classes with no significant P-value are displayed in
625 grey colour for comparative purposes (note that the class *Generation of Precursor Metabolites*
626 *and Energy* has no representation in the group of proteins increased in obesity). It should be
627 pointed that PANTHER may attribute multiple classes to a given protein.

628

629 **Fig. 3.** TKT, ACY-1 and HSP70 expression in human omental adipose tissue. Standardized
630 abundance was determined by DeCyder analysis of 2D-DIGE data from non-obese and obese
631 fat samples. The (+) and (-) symbols indicate increased and decreased levels with respect to the
632 internal standard, respectively (A). Representative [Western Blot](#) analysis of TKT, ACY-1 and
633 HSP70 expression from non-obese and obese fat samples. The results were normalized for B-
634 actin density (B). Values for relative intensity obtained after densitometry of the bands are
635 means +/- SD (C). Representative images of four independent analyses.

636

637 **Fig. 4.** Immunofluorescence staining of TKT in human omental adipose tissue, human pre-
638 adipocytes and human adipocytes differentiated *in vitro*. In fat biopsies TKT is mainly shown in
639 the cytosol of adipocytes and other SVF cells, but is also observed in the nuclei of a few cells
640 (four white circles) (A). In human pre-adipocytes TKT is shown both in the cytoplasm and the
641 nucleus (B). In human differentiated adipocytes TKT is exclusively observed in the cytosol (C).

642 Images are representative of adipose tissue sections collected from three subjects (A) and three
643 replicates (B and C).

644

645 **Fig. 5.** Immunofluorescence staining of ACY-1 in human omental pre-adipocytes and
646 adipocytes differentiated *in vitro*. ACY-1 (in red) is shown in the nucleus of pre-adipocytes (day
647 0) (A). In differentiated adipocytes (day 14) ACY-1 is observed in the cytosol and to a lesser
648 extent in the nucleus (B). Close-up view of a differentiated adipocyte showing ACY-1 around
649 the lipid droplets (C). The counterstaining of nuclei (DAPI) is shown in blue. Lipid droplets
650 have been stained with BODIPY 493/503 (in green). Images are representative of three
651 replicates.

652

653 **Fig. 6.** TKT and ACY-1 and protein levels assessed by Western Blot during adipogenic
654 maturation of 3T3-L1 (A). Values for relative intensity obtained after densitometry of the bands
655 are means \pm SD. * $p < 0.005$ and ** $p < 0.05$ for comparisons between TKT, and ACY-1 levels
656 at day 9 vs. day 0, respectively (B). FABP4 was used as an adipogenesis control. The results
657 were normalized for GAPDH density. Representative images of three independent analyses.

658

659 **Table 1.** Proteins identified by MALDI-MS showing significantly regulated expression in the
660 omental fat from morbidly obese ($n=6$) and non-obese ($n=6$) individuals. Protein identification
661 details are illustrated in Supplemental Table 1.

Table 1. Proteins identified by MALDI-MS showing significantly regulated expression in the omental fat from obese and non-obese individuals

DIGE			Protein			Mascot						
Spot ^a	p-value ^b	Av. Ratio ^c	Accession ^d	Locus ^e	Name	Score ^f	Expect ^g	ions score ^h	kDa theor ⁱ	pI theor ^j	Match pept ^k	Cover % ^l
1	1.6E-02	1.6	gi 642534	AAA85268	lumican	191	7.90E-13	102	38.7	6.2	5	14
2	8.0E-03	-2.0	gi 62414289	NP_003371	vimentin	273	4.90E-21		53.7	5.1	19	47
3	1.2E-02	2.2	gi 24234699	NP_002267	keratin 19	372	6.30E-31	157	44.1	5.0	18	43
4	1.4E-02	2.2	gi 42734430	NP_036364	polymerase I and transcript release factor	164	3.90E-10	73	43.5	5.5	6	12
5	3.9E-03	2.2	gi 24234699	NP_002267	keratin 19	257	2.00E-19		44.1	5.0	16	39
6	3.0E-02	1.7	gi 42734430	NP_036364	polymerase I and transcript release factor	129	2.80E-08	82	43.5	5.5	6	11
			gi 18645167	AAH23990	annexin A2	136	5.60E-09		38.8	7.6	9	28
7	2.1E-02	1.5	gi 167614506	NP_002289	L-plastin	235	3.10E-17	137	70.8	5.3	11	22
			gi 2897116	AAC39708	integrin alpha-7	157	2.00E-09	85	125.4	5.6	9	10
			gi 50415798	AAH78178	lamin-B1	129	1.20E-06		38.3	5.4	9	30
8	3.6E-02	2.1	gi 55962552	CAI18465	heat shock 70kDa protein 1A	181	7.80E-12	85	52.2	5.4	7	13
9	3.3E-02	1.7	gi 3127926	CAA36267	collagen type VI, alpha 3 chain	89	1.30E-02	91	345.1	6.4	8	3
10	4.2E-02	1.6	gi 42734430	NP_036364	polymerase I and transcript release factor	157	2.00E-09	74	43.5	5.5	6	12
11	5.5E-03	1.8	gi 62898171	BAD97025	L-plastin variant	240	9.90E-18	103	70.8	5.2	10	18
12	2.0E-02	1.6	gi 119626083	EAX05678	albumin, isoform CRA_t	168	1.60E-10		60.2	6.7	14	27
13	1.2E-02	2.1	gi 4506925	NP_003013	SH3 domain binding glutamic acid-rich protein like	87	4.60E-04	69	12.8	5.2	1	8
14	4.7E-02	1.4	gi 42476013	NP_940916	NHL repeat containing 2	114	3.90E-05	57	80.2	5.3	4	6
15	6.3E-03	2.6	gi 25777732	NP_000681	mitochondrial aldehyde dehydrogenase 2 precursor	312	6.30E-25	149	56.9	6.6	11	22
16	4.3E-02	-1.5	gi 91992949	AAI14619	guanine nucleotide-binding protein beta subunit	161	7.80E-10	77	37.1	5.6	5	16
17	3.0E-02	1.6	gi 181573	AAA35763	cytokeratin 8	147	2.00E-08	82	53.5	5.5	5	10
18	5.8E-05	2.7	gi 67782365	NP_005547	keratin 7	293	4.90E-23	133	51.4	5.4	15	30
19	2.2E-02	1.5	gi 4096652	AAC99987	aryl sulfotransferase	102	1.40E-05	82	34.3	5.8	1	3
20	2.7E-03	2.0	gi 2781209	1FZA_C	fibrinogen gamma chain	240	9.90E-18	128	36.5	5.9	7	33
21	3.1E-02	1.9	gi 4502101	NP_000691	annexin I	187	2.00E-12	105	38.9	6.6	5	17
22	3.1E-03	2.3	gi 1419564	CAA67203	keratin 8, isoform CRA_d	115	3.10E-05	81	30.8	5.0	2	8
23	1.9E-03	-2.1	gi 662841	AAA62175	heat shock protein 27	126	2.50E-06	93	22.4	7.8	2	13
24	4.8E-03	1.6	gi 168988718	2VDB_A	serum albumin	161	7.80E-10		67.8	5.6	12	18
25	1.1E-03	2.6	gi 237823916	3GHG_C	chain C, human fibrinogen	396	2.50E-33	216	47.0	5.5	14	51
			gi 181573	AAA35763	cytokeratin 8	366	2.50E-30	186	53.5	5.5	15	37
26	2.0E-03	-1.5	gi 4501901	NP_000657	aminoacylase 1	190	9.90E-13	60	46.1	5.8	8	18
27	1.6E-03	1.5	gi 18645167	AAH23990	annexin A2	185	3.10E-12	69	38.8	7.6	7	20
28	1.2E-02	-1.7	gi 4557581	NP_001435	fatty acid binding protein 5 (psoriasis-associated)	277	2.00E-21	88	15.5	6.6	10	68
29	3.1E-02	1.8	gi 119626066	EAX05661	albumin, isoform CRA_c	141	7.80E-08		27.7	6.4	8	30
30	4.4E-02	1.9	gi 17389815	AAH17917	triosephosphate isomerase 1	131	7.80E-07		26.9	6.5	7	32
31	1.1E-02	-1.7	gi 4557233	NP_000008	short-chain acyl-CoA dehydrogenase precursor	119	1.20E-05		44.6	8.1	7	19
32	3.9E-02	-1.5	gi 157168362	NP_000261	nucleoside phosphorylase	123	4.90E-06	71	32.3	6.5	3	14
33	3.1E-02	-1.8	gi 110590599	2HAV_A	serotransferrin precursor	119	1.20E-05		77.0	6.9	9	17
34	6.3E-03	-1.5	gi 4557231	NP_000007	medium-chain acyl-CoA dehydrogenase isoform a precursor	158	1.60E-09	91	47.0	8.6	4	13
35	2.1E-03	-1.5	gi 119631279	EAX10874	3-hydroxyisobutyryl-coenzyme A hydrolase, isoform CRA_b	159	1.20E-09		49.4	9.4	9	23
36	3.2E-02	-1.5	gi 4502517	NP_001729	carbonic anhydrase I	141	1.80E-09	72	28.9	6.6	4	21
37	3.1E-02	1.4	gi 27436946	NP_733821	lamin A/C isoform 1 precursor	521	7.80E-46	232	74.4	6.6	22	38

38	4.8E-03	-1.5	gij119590499	EAW70093	fumarate hidratase, isoform CRA_d	174	3.90E-11	136	46.6	6.9	2	6
39	6.0E-05	-1.7	gij189181759	NP_001121188	electron transfer flavoprotein, alpha polypeptide isoform b	167	4.50E-12	93	30.2	8.8	4	20
40	4.3E-02	-1.4	gij4557735	NP_000231	monoamine oxidase A	126	2.50E-06	69	60.2	7.9	4	11
41	1.0E-02	-1.9	gij30583667	AAP36082	citrate synthase	109	1.20E-04	69	29.6	7.8	2	7
42	1.1E-02	-1.6	gij71296885	AAH44787	monoamine oxidase A	146	2.50E-08	66	60.2	6.9	6	15
43	4.3E-03	-1.4	gij4557014	NP_001743	catalase	278	1.60E-21	115	59.9	6.9	9	25
44	1.3E-02	-1.5	gij388891	AAA61222	transketolase	150	9.90E-09		68.5	7.9	10	22
45	3.2E-03	-1.6	gij179950	AAB59522	catalase	195	3.10E-13	70	51.6	7.8	8	23
46	3.1E-03	-1.8	gij223002	0401173A	fibrin beta	170	9.90E-11		51.4	8.0	10	26
47	1.4E-04	-1.6	gij48257138	AAH00105	citrate synthase, mitochondrial precursor	211	7.80E-15	101	45.8	6.5	6	16
48	3.0E-02	-1.7	gij388891	AAA61222	transketolase	178	1.60E-11		68.5	7.9	10	25
49	1.0E-02	-1.5	gij4557237	NP_000010	acetyl-coenzyme A acetyltransferase 1 precursor	129	1.20E-06	95	45.5	9.0	2	7
50	8.8E-03	-1.5	gij13111901	AAH03119	ATP synthase subunit alpha, mitochondrial precursor	101	1.80E-05	86	40.4	8.9	1	3
51	1.8E-02	-1.4	gij16950633	NP_446464	argininosuccinate synthetase 1	180	9.90E-12	49	46.8	8.1	7	16
52	1.7E-02	-1.7	gij598143	AAB48003	alcohol dehydrogenase beta-3 subunit	220	9.90E-16		40.7	8.5	12	32
53	2.7E-02	2.0	gij4757756	NP_004030	annexin A2 isoform 2	283	4.90E-22	87	38.8	7.6	15	48
54	1.9E-02	-1.6	gij119626625	EAX06220	L-3-hydroxyacyl-coenzyme A dehydrogenase short chain isoform CRA_c	170	9.90E-11	78	7.6	9.3	4	52
55	4.1E-02	-1.6	gij598143	AAB48003	alcohol dehydrogenase beta-3 subunit	212	6.20E-15	63	40.7	8.5	10	28
56	3.5E-02	-1.5	gij40807491	NP_001986	acyl-CoA synthetase long-chain family member 1	306	2.50E-24	141	78.9	6.8	11	20

^aSpot numbering as shown in 2-DE silver gel in Figure 1. ^b*p*-value of the Student t-test and ^caverage volumen ratio (Obese/non-Obese) as calculated by the DeCyder analysis. ^dProtein and ^elocus accesion codes from the NCBI database. ^fMascot score, ^gMascot expected value, ^hMascot Ions score, ⁱtheoretical molecular weight (kDa) and ^j*pI*, ^knumber of matched peptides and ^lprotein sequence coverage for the most probable candidate as provided by Mascot. Protein identification details (MS and MS/MS spectra) are listed in Supplemental Table 1.

Fig.1 Silver stained 2D-gel
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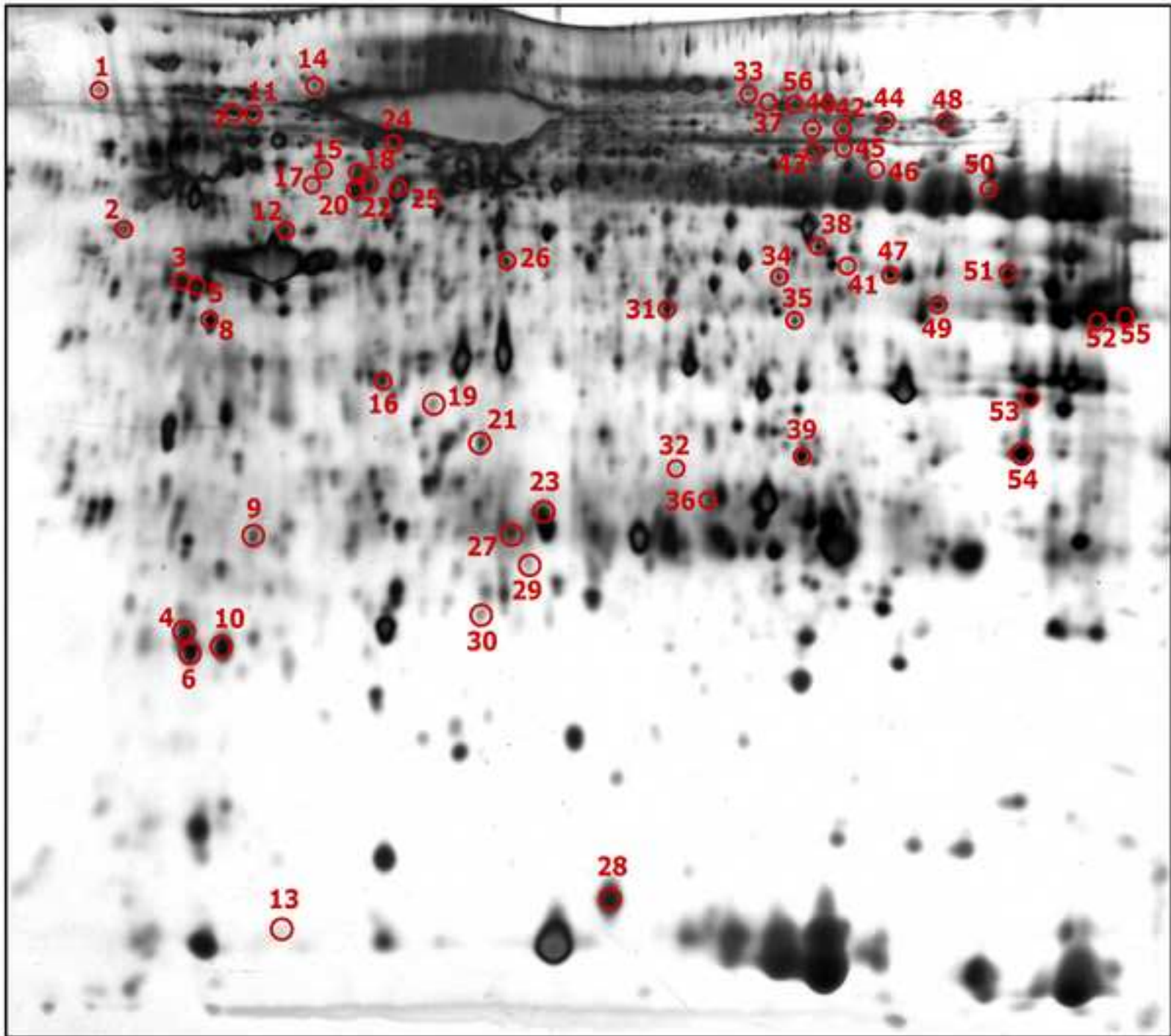
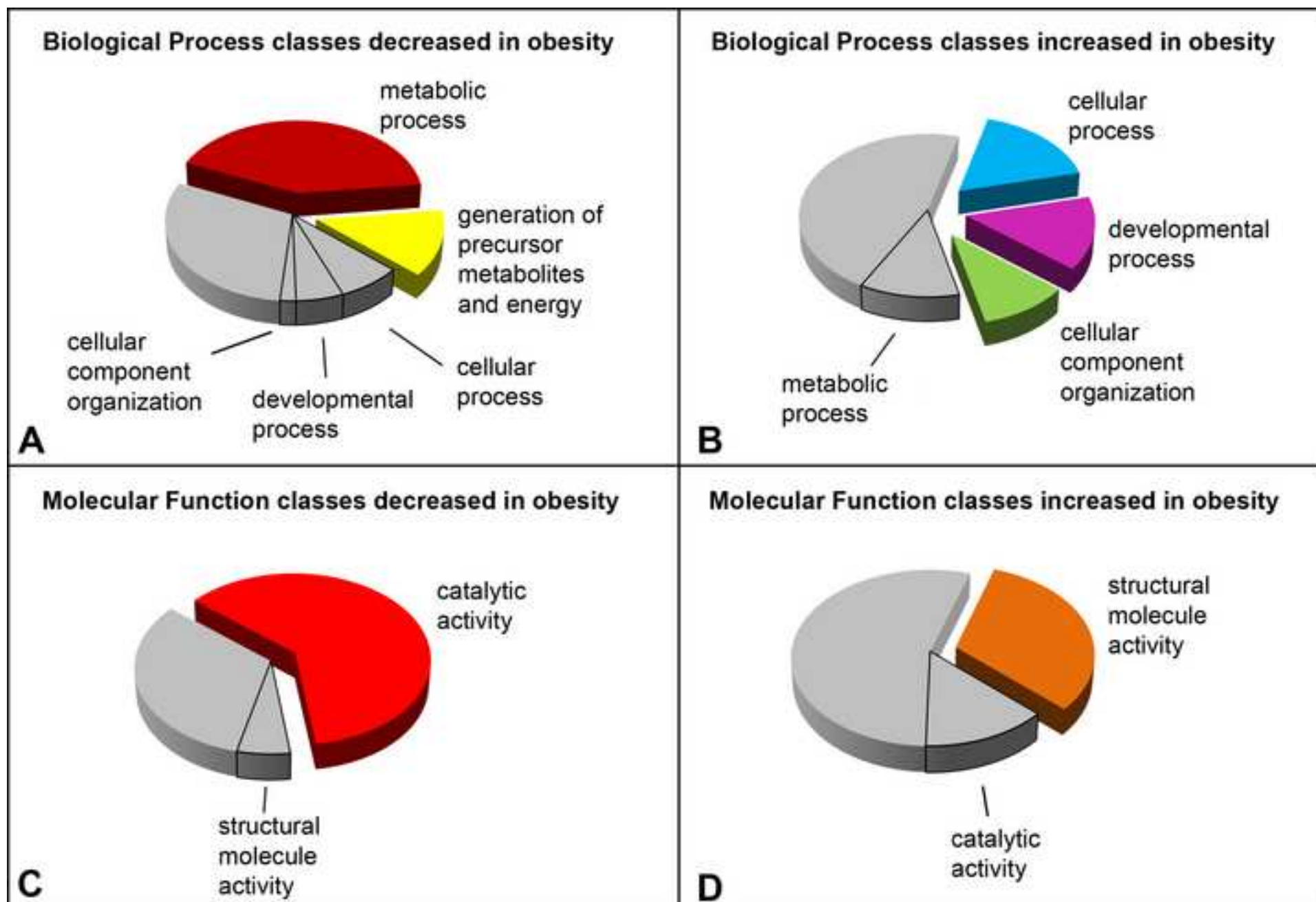


Fig2. Pie chart representations of PANTHER
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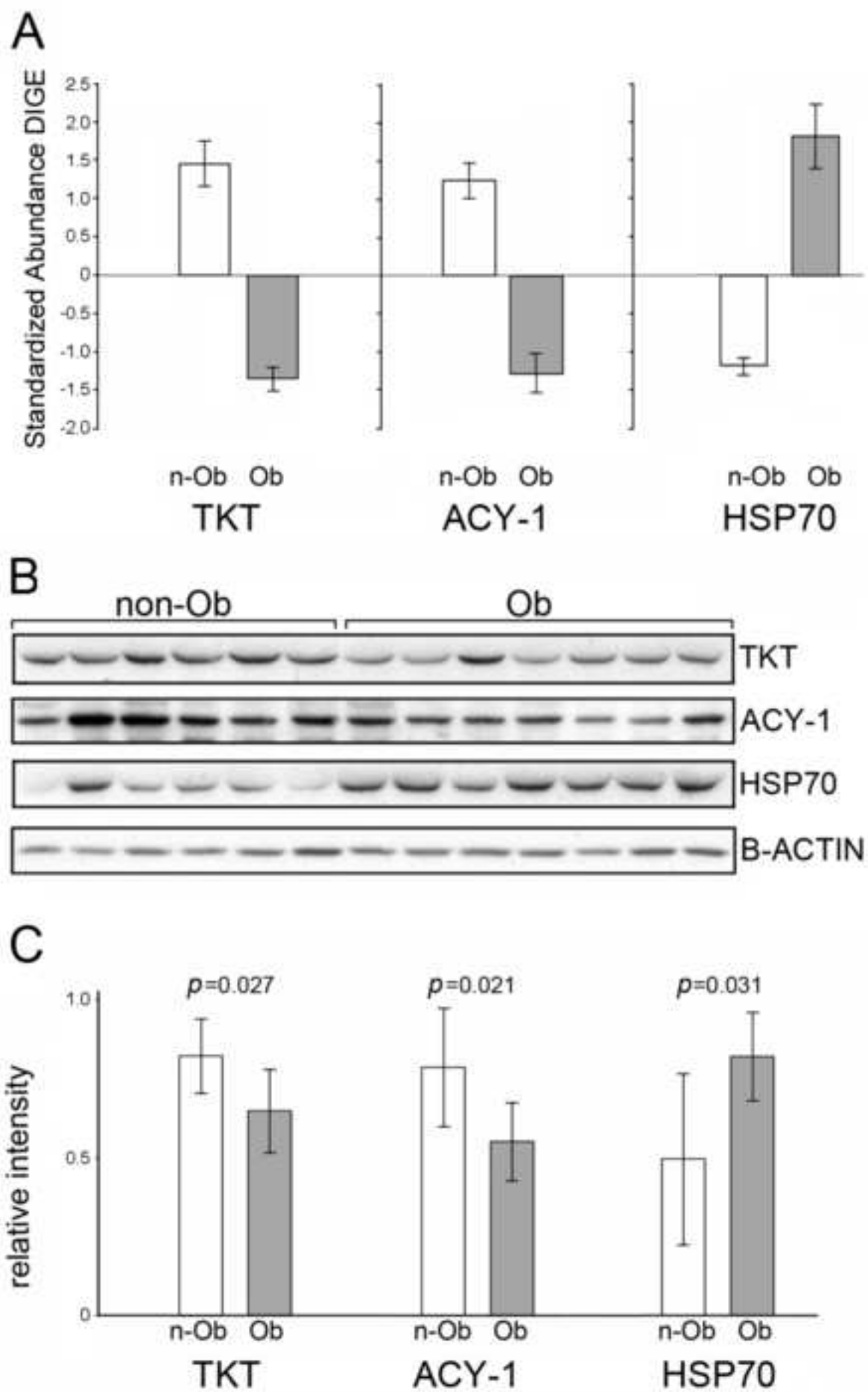
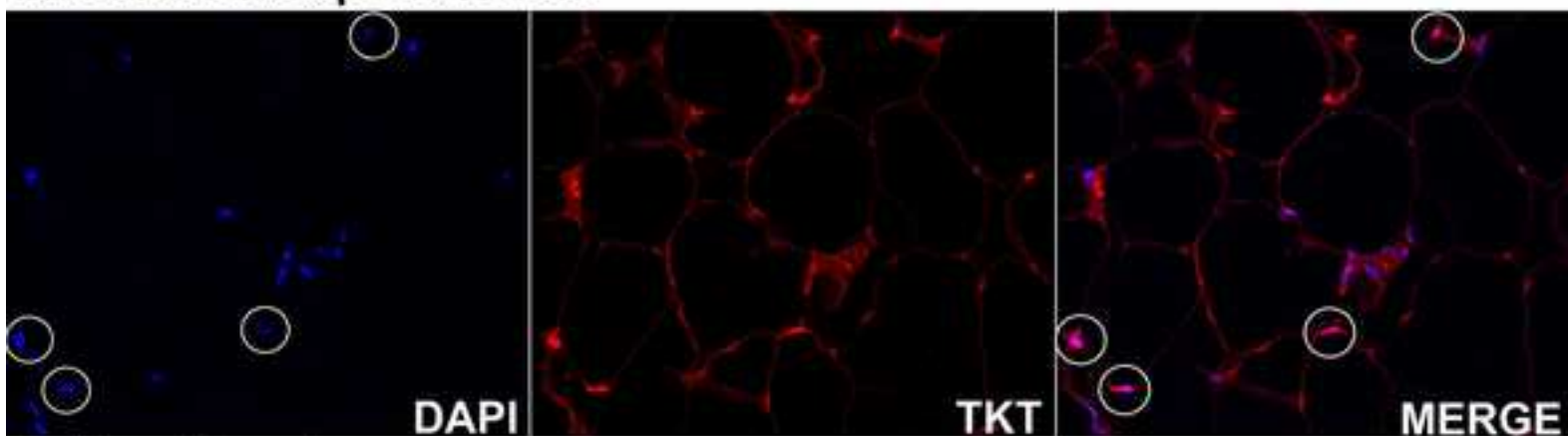
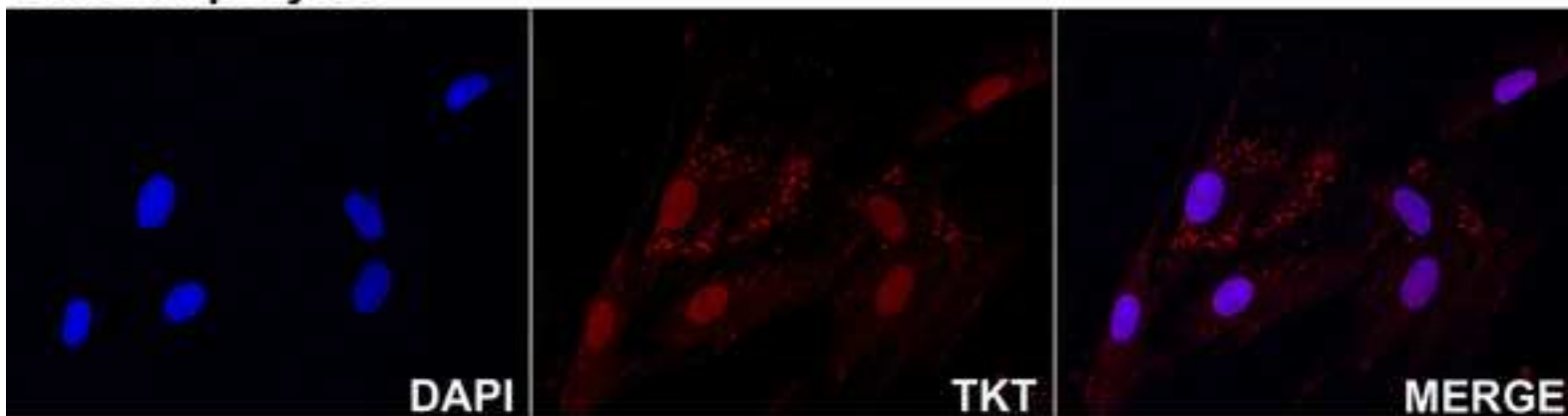


Fig4. Immunofluorescence staining of TKT
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A. Omental Adipose Tissue



B. Preadipocytes



C. Adipocytes

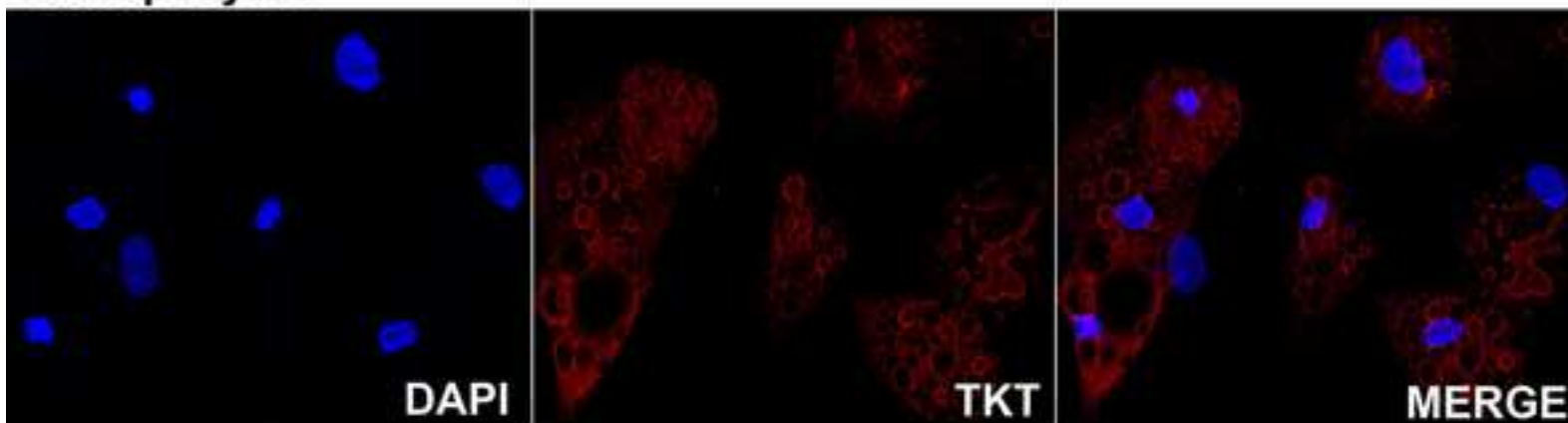
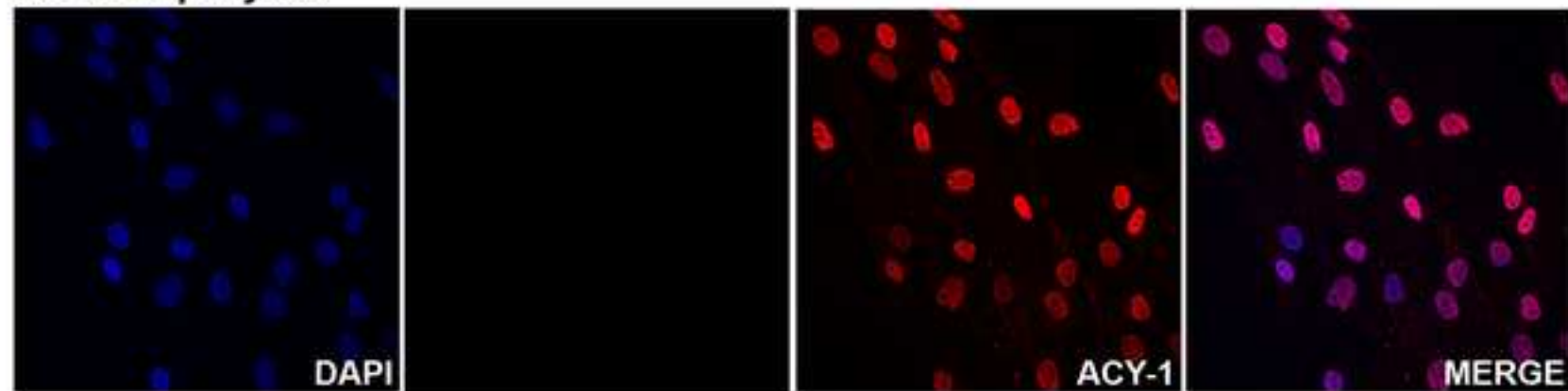
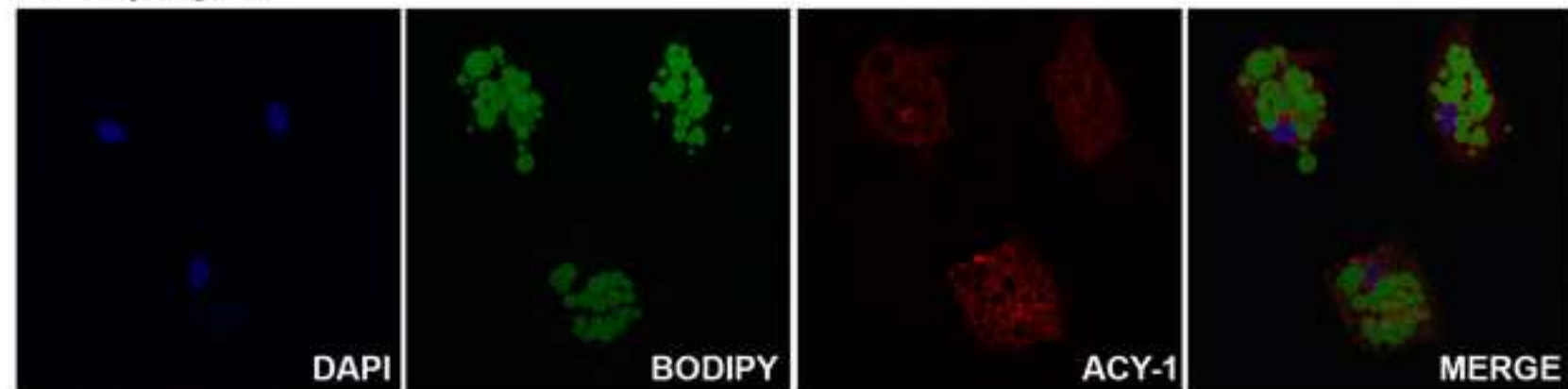


Fig5. Immunofluorescence staining of ACY-1
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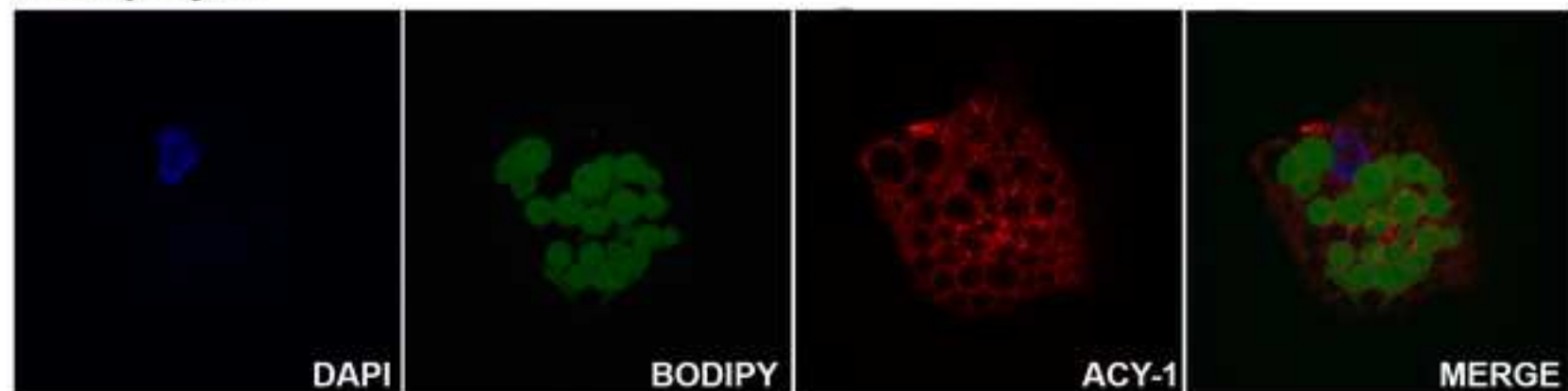
A. Preadipocytes

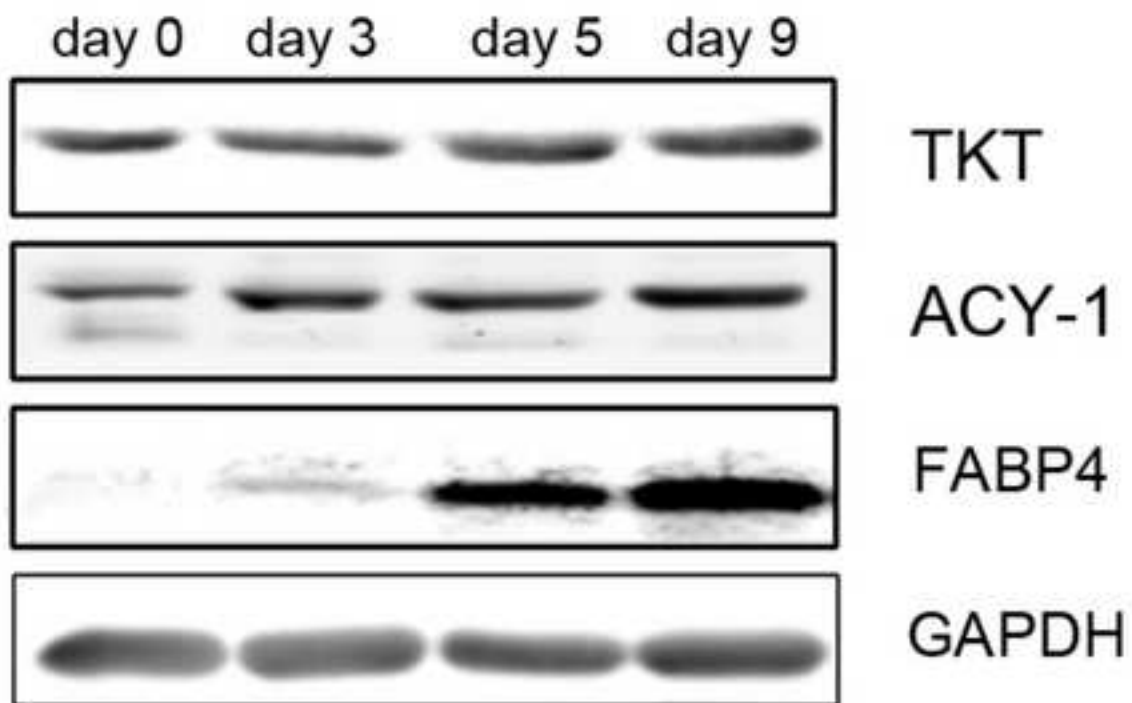
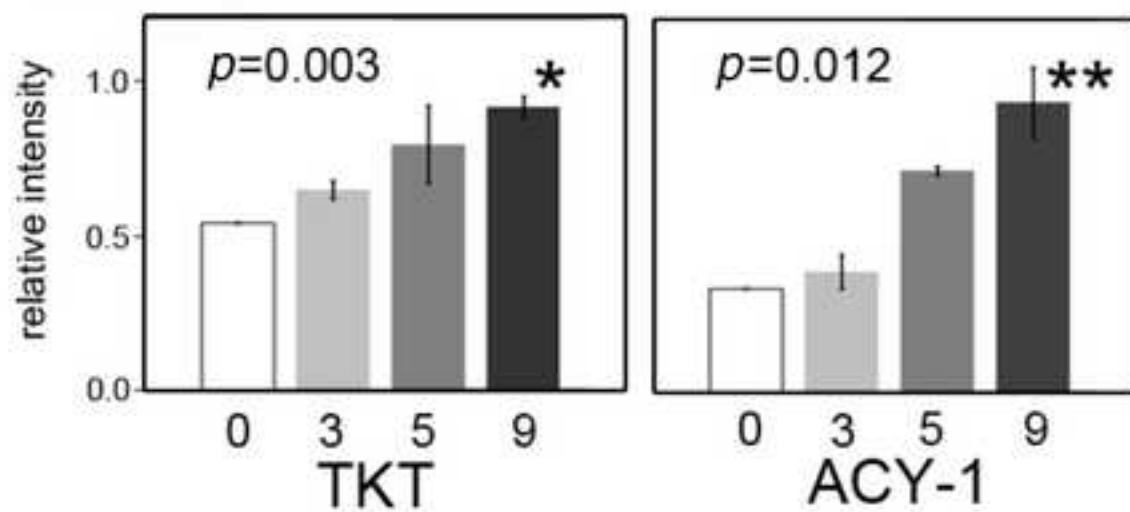


B. Adipocytes



C. Adipocytes



A**B**

Supplementary material

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Supplementary Table 1

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New Supplementary Table 2

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